PROGRESS IN MEDICINAL CHEMISTRY 26

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G. B. WEST
EDITORS

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Preface

We have pleasure in presenting six reviews in this volume, five of which cover important advances in the chemistry and biology of medicinal products. Three of the reviews concern compounds which are currently being evaluated for their efficacy in the treatment of cancer.

In Chapter 1, an exhaustive survey is presented of the large number of analogues of methotrexate as antifolates. These compounds selectively destroy malignant cells without causing appreciable injury to normal host tissues. In the next two chapters, the treatment of cancer with hormones is discussed. The biological significance of aromatase activity in breast cancer is reviewed in Chapter 2 and the value of analogues of luteinizing hormone-releasing hormone in the treatment of prostate cancer is assessed in Chapter 3.

An application of microcomputers in biochemistry, described in Chapter 4, concerns their use in the education of medical and science students and an example of their use in one medical school is given. In Chapter 5, the value of NMR spectroscopy for structural and stereochemical analysis of medicinal compounds is analysed; considerable progress has recently been made in the use of carbon-13 spectroscopy and of solid samples.

Finally, Chapter 6 is a survey of biologically active copper complexes in inflammatory, cancerous, diabetic and other diseases. This review updates one which appeared in Volume 15.

We thank our authors for surveying and summarizing the literature of each topic. We also offer our thanks to owners of copyright material which we have included, and to the staff of our publishers for their continuing help and encouragement.

G.P. Ellis
July 1988 G.B. West

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Progress in Medicinal Chemistry – Vol. 26, edited by G.P. Ellis and G.B. West

1 Chemistry and Biological Activity of Antifolates

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INTRODUCTION

The synthesis of methotrexate (MTX, *Figure 1.1*) in 1947 by chemists at Lederle Laboratories [1] was followed almost at once by widespread recognition of its clinical potential as a cancer remedy [2]. Not surprisingly, medicinal chemists soon became drawn to this multifunctional molecule as an object on which to practice their time-honoured (and sometimes maligned) strategy of 'enlightened empiricism'. In considering the many structural modifications that have been made over the past four decades with the aim of qualitatively or quantitatively altering the therapeutic spectrum of MTX, the molecule may be conveniently dissected into seven regions, as indicated in *Figure 1.1* and summarized below.

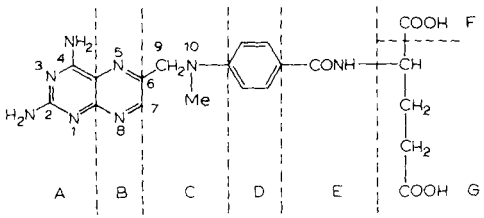


Figure 1.1. Seven regions of MTX that have been subjected to structure modification

Notable examples of changes in *Region A* include C-for-N substitution at positions 1 or 3 and replacement of the amino group at position 4 by a methylthio group. These studies led to the accepted view that the 2,4-diaminopyrimidine moiety is sacrosanct for antitumour activity when the latter is based on inhibition of dihydrofolate reductase (DHFR).

Region B, comprising the pyrazine moiety, has provided a more fertile field for exploration. One permissible change in the pyrazine ring has been found to involve C-for-N substitution at positions 5 and 8. Substitition of N for C at position 7 and replacement of the hydrogen at position 7 by a methyl group have also been accomplished, but have resulted in complete loss of antitumour activity. More successful examples of Region B modification are the quinazoline (5,8-dideaza) analogues, of which the most prominent 2,4-diamino derivatives to date have been methasquin and chlorasquin. An important

addition to the quinazoline group has also been CB3717, a 2-amino-4 oxo rather than 2,4-diamino derivative additionally modified by propargyl for methyl substitution at position 10. As a result of these substantial structure changes, the antifolate action of CB3717 was redirected from DHFR to thymidylate synthase (TS) as the target enzyme.

Region C, usually referred to as the 'bridge region', has been targeted as another site of major interest. The concept that changes in this part of the molecule should be vigorously explored was articulated some time ago, and was reduced to practice eventually in several forms, including insertion of extra CH_2 spacers or an extra nitrogen atom, interchange of C^9 and N^{10}, and replacement of N^{10} by oxygen or sulphur. Analogues of MTX with the methyl group at N^{10} replaced by other carbon-containing groups have also been described. Most recently, another important series of analogues was developed in which N^{10} was replaced by carbon in the form of either a simple CH_2 group or a branched-chain carbon. At the present time, a member of this latter group, 10-ethyl- 10-deazaAMT, is in active clinical trial and appears to be superior to MTX in several respects. Combined modification of Regions B and C has also been achieved successfully in the form of 5,10-dideaza, 8,10-dideaza and 5,8,10-trideaza analogues. The 5,10-dideaza analogue of tetrahydrofolic acid in particular is reported to be a potent inhibitor of *de novo* purine synthesis at the level of the enzyme glycinamide ribonucleotide formyltransferase (GAR transformylase). There is currently strong interest in antifolates of this type because of their potential ability to complement classical therapeutic approaches based on DHFR or TS inhibition.

The phenyl ring, comprising *Region D*, has been halogenated at the 3′ and 5′ positions (*ortho* to N^{10}), as well as the 2′ and 4′ positions (*meta* to N^{10}), and also has been replaced by heterocyclic rings. However, with the exception of 3′,5′-dichloromethotrexate (DCM), which continues to be of interest because of its enhanced lipophilicity and more efficient hepatic extraction relative to MTX, modification of Region D has been only marginally useful.

Attempts to modify *Region E*, comprising the amide bond between the pteroyl moiety and amino acid side chain have not been profitable. For exemple, insertion of extra CH_2 groups and reduction of the C=O group to CH_2 have both led to inactive compounds.

Likewise not amenable thus far to structural modification has been the α-carboxyl group (*Region F*), which appears to contribute critically not only to DHFR binding but also to transport across the cell membrane and subsequent γ-polyglutamylation inside the cell.

The side-chain of the glutamate moiety exclusive of the α-carboxyl group (*Region G*), on the other hand, has shown itself to be a domain where structur-

al change is relatively well tolerated. This part of the molecule has attracted particular attention over the past several years because of its relevance to γ-polyglutamylation, a major determinant of the antitumour activity and therapeutic selectivity of MTX and other 'classical antifols'. Modifications that have been made in this part of the molecule include esterification and amidation of the γ-COOH group, insertion of extra CH_2 groups between the carboxyls, replacement of the γ-COOH group by a γ-SO_3H or γ-PO_3H_2 group, and introduction of chemically reactive groups capable of inactivating an enzyme by covalent bond formation at, or near, the active site.

This review is an attempt to give a comprehensive account of structural changes that have been made in each of the above regions of MTX. While some attention will be given to the synthetic chemical methods used in preparing MTX analogues, (which have been reviewed [2a]), a major goal will be to also provide an overview of the fascinating structure-activity correlations made possible by the availability of these compounds. Because published biochemical and pharmacological data on MTX analogues are at this point extremely voluminous, and because only a fraction of this information can be presented here, the reader is advised to consult the original literature for additional details. For a remarkable account of the early history of MTX and other classical antifolates, reference to a recent article [3] by a pioneer in this field is highly recommended. Other contemporary works suggested for reading are a 1982 review on 2,4-diaminopyrimidines by Roth and Cheng [4] and a 1984 monograph on folate antagonists as therapeutic agents, edited by Sirotnak, Burchall, Ensminger and Montgomery [5].

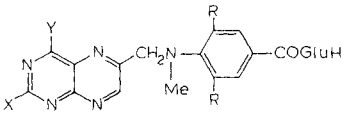
CHANGES IN REGION A

MODIFICATIONS AT POSITIONS 2 AND 4

An early indication that 2,4-diamino substitution in the pteridine ring of MTX contributes in a major way to microbiological activity against the test organism *Streptococcus faecium* was the finding by the Lederle group [6] of a marked decrease in growth inhibition by the 4-*N*-methylamino, 4-*N*,*N*-dimethylamino, and 4-piperidino analogues (II.1)–(II.3*) and the 2-*N*,*N*-

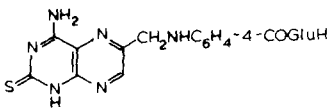
* The following abbreviations are used in this chapter:
GluH = $NHCH(COOH)(CH_2)_2COOH$;
GluR = $NHCH(COOR)(CH_2)_2COOR$;
where R = Me, Et, etc.

dimethylamino analogue (II.4). The synthesis of compounds (II.1)–(II.3) was accomplished by condensing the appropriate N-substituted 2,4,5,6-tetraaminopyrimidine with *N*-(4-aminobenzoyl)-L-glutamic acid and 2,3-dibromopropionaldehyde, in what has come to be known as the Waller synthesis. The pyrimidines were prepared from 2,4-diaminopyridin-6(2*H*)-one by chlorination with phosphoryl chloride, amination with methylamine, dimethylamine or piperidine, and reduction with sodium dithionite. The DCM analogues (II.5) and (II.6), synthesized from (II.3) and (II.4), respectively, by chlorination in glacial acetic acid were likewise devoid of activity against *S. faecium*. The 2-substituted analogue (II.4) was synthesized from 4,5,6-triamino-2-*N*,*N*-dimethylaminopyrimidine, which was obtained from 4,6-diamino-2-*N*,*N*-dimethylaminopyrimidine by nitrosation and reduction.



(II.1) X = NH_2, Y = NHMe, R = H
(II.2) X = NH_2, Y = NMe_2, R = H
(II.3) X = NH_2, Y = piperidino, R = H
(II.4) X = NMe_2, Y = NH_2, R = H
(II.5) X = NH_2, Y = piperidino, R = Cl
(II.6) X = NMe_2, Y = NH_2, R = Cl

Evidence of the critical importance of a 2-amino group in the pteridine moiety was also furnished by De Clercq and Truhaut [7], who synthesized *N*-{4-[*N*-(4-amino-2(1*H*)-thiono-6-pteridinyl)methyl]amino}benzoyl-L-glutamic acid (II.7) and found it to have an LD_{50} value (2000 mg/kg) in Swiss mice roughly 1000-fold higher than that of aminopterin (AMT). The synthesis began with 4,6-diaminopyrimidin-2(1*H*)-thione, which was nitrosated at position 5, reduced with sodium hydrosulphide, and then *S*-methylated. The resultant product was converted via the Waller synthesis to the AMT analogue (II.8), which contains a methylthio group in place of the amino group at position 2. Reaction of (II.8) with sodium hydrosulphide afforded (II.7).



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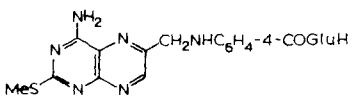
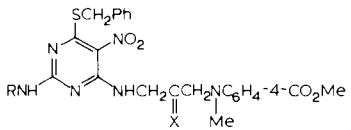
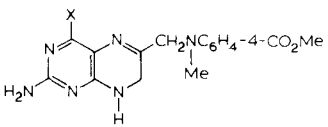


(II.8)

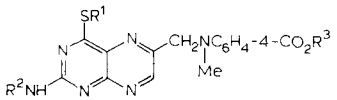
Analogues of MTX with a sulphur atom at position 4 rather than 2 were synthesized by Elliott *et al.* [8,9]. In one synthetic scheme [8], 2-acetamido-6-benzylthio-4-chloro-5-nitropyrimidine was obtained in several steps from 2-amino-4-chloro-5-nitropyrimidin-6(1*H*)-one and elaborated to the oxime (II.9) and ketone (II.10), the last of which was cyclized by warming to 50 °C in the presence of Raney nickel to form the dihydropteridine (II.11). After gentle oxidation to (II.13) with potassium permanganate, treatment with sodium hydrosulphide led to the 2-aminopteridine-4(3*H*)-thione (II.15), which was converted via a series of straightforward reactions to the 2-acetamido-4-methylthiopteridine (II.14). Coupling of (II.14) to diethyl L-glutamate by the mixed carboxylic-carbonic anhydride method, using isobutyl chloroformate and triethylamine, gave a 65% yield of the protected adduct (II.18), which upon successive reactions with sodium hydrosulphide and sodium hydroxide was converted to N^{10}-methyl-4-thiofolic acid (II.17). *S*-Alkylation of (II.17) with methyl iodide in the presence of base led to the 2-amino-4-methylthio derivative (II.19). The UV spectra of (II.17) [λ_{max} (0.1 M NaOH) 300 mm (ε 27,600), 410 (10,400); λ_{max} (0.1 M HCl) 300 nm (ε 21,100), 378 (10,500)] (5) were consistent with its 4-thione structure. The NMR spectrum, in d_6-DMSO, showed the C-7 proton as a singlet at δ 8.58, and the phenyl protons as a pair of doublets (A_2B_2 pattern) at δ 6.81 and 7.73.



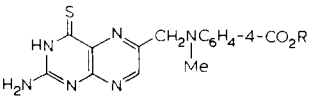
(II.9) X = NOH, R = Ac
(II.10) X = O, R = H



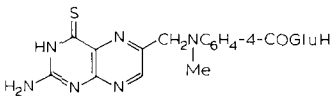
(II.11) X = SCH_2Ph
(II.12) X = Cl



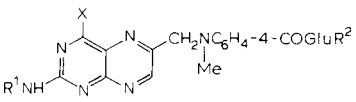
(II.13) R^1 = CH_2Ph, R^2 = H, R^3 = Me
(II.14) R^1 = Me, R^2 = Ac, R^3 = H



(II.15) R = Me
(II.16) R = H

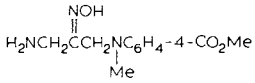


(II.17)



(II.18) X = SMe, R^1 = Ac, R^2 = Et
(II.19) X = SMe, R^1 = R^2 = H
(II.20) X = $NHNH_2$, R^1 = R^2 = H

In a subsequent variation of the above route, Elliott *et al.* [9] condensed 2,5-diamino-4,6-dichloropyrimidine with the oxime (II.21), in a very slow reaction requiring 2 weeks at 65 °C, and isolated the relatively stable dihydropteridine (II.12). The unusual stability of this product was attributed to the electron-withdrawing influence of the 4-chloro substituent. Successive reactions with sodium hydrosulphide and sodium hydroxide converted (II.12) to the sulphur analogue (II.16) of N^{10}-methylpteroic acid. After *S*-methylation and protection of the 2-amino group by acetylation, mixed anhydride coupling to diethyl L-glutamate was performed. The yield in this reaction was only 22% as compared with the 65% recorded for the 4-*S*-benzyl analogue. Treatment of (II.18) with sodium hydrosulphide and sodium hydroxide afforded (II.17), and alkylation of (II.17) with methyl iodide gave (II.19). An interesting transformation of (II.19) also reported in this work involved reaction with hydrazine to form the 4-hydrazino analogue (II.20) of MTX. This is a novel structural modification of Region A whose potential has thus far been explored only minimally.



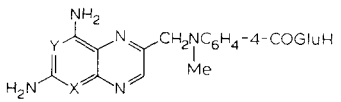
(II 21)

The activity of compounds (II.17), (II.19) and (II.20) as DHFR inhibitors and as inhibitors of cell growth in culture was evaluated [9]. Anti-DHFR activity *in vitro* was measured with partially purified enzyme from pigeon liver, and was found to be reduced more than 1000-fold in comparison with MTX. In the *S. faecium* growth assay, however, (II.17) (IC_{50} = 5.2 nM) was only 7-fold less potent than MTX (IC_{50} = 0.7 nM), while (II.19) (IC_{50} = 24 nM) was only 34-fold less potent. These findings suggest that (II.17) and (II.19) may be better inhibitors of the bacterial than of the avian enzyme, or that they may exert their antiproliferative effect on *S. faecium* via mechanisms other than, or in addition to, DHFR inhibition. Despite their antibacterial activity, compounds (II.17) and (II.19) failed to affect the growth of cultured human epidermal carcinoma (KB) cells significantly. On the other hand, the 4-hydrazino compound (II.20) did show a very modest level of activity against the mammalian cells, with an IC_{50} value of approximately 8 μM.

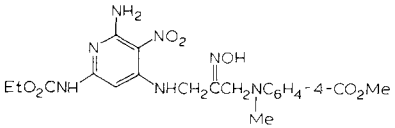
1-DEAZA AND 3-DEAZA COMPOUNDS

Elegant routes to the 1-deaza analogue (II.22) and 3-deaza analogue (II.23) were developed by Elliott *et al.* [10] with the aim of assessing the relative

importance of the ring nitrogens at positions 3 and 1 as determinants of biological activity. Access to (II.22) was gained via an adaptation of the classical Boon-Leigh pteridine synthesis [11]. 2-Amino-4-chloro-6-ethoxycarbonylamino-3-nitropyridine was converted to the oxime (II.24), and the latter was hydrogenated in the presence of Raney nickel to obtain a mixture of the dihydropyrimidopyrazine (II.25) and a minor amount of the uncyclized byproduct (II.26). Alternatively, (II.25) could be prepared from (II.24) by preliminary acidolysis of the oxime group followed by reductive ring closure, though this route proved less satisfactory upon scale-up. Mild oxidation with $KMnO_4$ and ester and urethane hydrolysis with KOH in ethanol converted (II.25) to the fully aromatic acid (II.27), which, after amino-group protection by acetylation, was coupled to diethyl L-glutamate with the aid of dicyclohexylcarbodiimide in pyridine. Simultaneous removal of the ester and amide blocking groups to form (II.22) was achieved by hydrolyzing the coupling product with NaOH in refluxing ethanol (vigorous conditions under which there might be at least partial racemization of the glutamate side-chain). The ultraviolet absorption spectrum of (II.22) [λ_{max} (0.1 M HCl) 245 nm (ε 22,800), 321 (29,800), 427 (br, 3,800); λ_{max} (0.1 M NaOH) 268 nm (ε 28,300), 306 (28,000), 427 (br, 3,800)] was, as expected, somewhat different from that of MTX. Aromatic protons on the pyridopyrazine ring system gave rise to NMR singlets at δ 5.99 (pyridine proton) and 8.62 (pyrazine proton). An interesting feature of (II.22) was its spectrophotometric pK_a of 4.5. Since MTX had a pK_a of 5.5, carbon for nitrogen replacement at position 1 produced a 10-fold loss in basicity which would be expected to be detrimental to DHFR binding. This was supported by measurements of the ability of compound (II.22) to inhibit enzyme from pigeon liver and *S. faecium*. With both enzymes, the 1-deaza analogue was a poorer inhibitor than MTX by at least two orders of magnitude, attesting to the importance of N^1 for tight binding to the enzyme. Compelling evidence for the critical role of N^1 has come also from direct studies of the interaction of MTX with DHFR by NMR spectrometry and X-ray crystallography. These studies have been reviewed in detail elsewhere [12].



(II.22) X = CH, Y = N
(II.23) X = N, Y = CH



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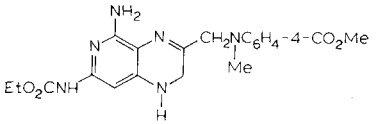

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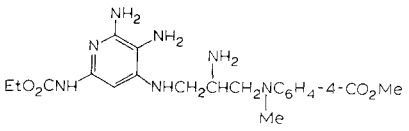

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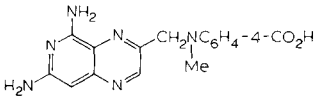

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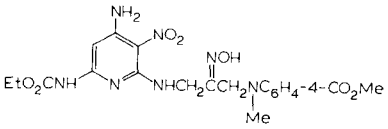

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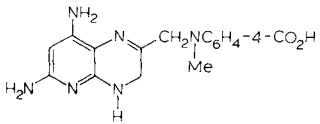

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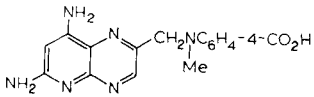


(II.30)

A scheme analogous to the one described above was followed [10] to prepare (II.23), the 3-deaza analogue of MTX. In this instance, 4-amino-2-chloro-6-ethoxycarbonylamino-3-nitropyridine was elaborated to the oxime (II.28), and the latter was cylized to the dihydropyridopyrazine (II.29) in the presence of Raney nickel. Compound (II.29) was then converted to the acid (II.30), which, on carbodiimide coupling to diethyl L-glutamate followed by vigorous alkaline treatment to remove the ester and amide protecting groups, gave (II.23). The ultraviolet spectrum of (II.23) [λ_{max} (0.1 M HCl) 223 nm (ε 49,100), 313 (br, 21,400), and 330 (br, 20,900); λ_{max} (0.1 M NaOH) 219 nm (ε 44,100), 262 (21,200), 305 (br, 25,800), 355 (br, 12,400)] was quite unlike that of the 1-deaza analogue (II.22) (see above). The NMR spectrum was also distinctive, with singlets at δ 6.07 (pyridine ring proton) and δ 8.52 (pyrazine ring proton). The spectrophotometric pK_a was found to be 7.4, indicating a > 1000-fold *increase* in basicity relative to (II.22). As might be expected from these results, (II.23) was considerably more active than (II.22) as a DHFR inhibitor, with an IC_{50} against purified pigeon liver enzyme only 5-fold lower than that of MTX. The 3-deaza analogue was also active against L1210 murine leukaemia in mice, producing a survival increase comparable to that of MTX, albeit at a higher dose. This increased dosage requirement is likely to be due to a combination of factors such as decreased DHFR binding, decreased cellular uptake, and possibly decreased intracellular polyglutamylation.

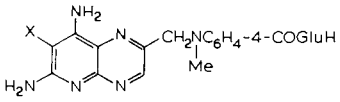
A more detailed analysis of the *in vitro* mode of action of (II.23) in comparison with AMT, MTX, and several MTX analogues was carried out by Sirotnak *et al.* [13]. The K_i of (II.23) as an inhibitor of DHFR isolated from L1210 murine leukaemia cells was 31 nM, whereas the K_i values of MTX and AMT were 3.2 and 4.3 pM, respectively. When inhibition of DHFR from *Diplococcus pneumoniae* was compared, on the other hand, (II.23) and MTX proved equipotent. Thus, deletion of N^3 resulted in no loss of affinity for the bacterial enzyme and only a small loss of affinity for the avian enzyme (see above), but a dramatic 7,000-fold loss of affinity for the enzyme from murine cells.

A comparison of the kinetics of uptake of (II.23), AMT, and MTX by L1210 cells showed that these compounds do not differ greatly in either their influx or efflux [13]. The K_m for influx of (II.23) (3 μM) was virtually identical with that of MTX and only 2.5-fold higher than that of AMT, while all three compounds showed essentially the same V_{max}. Likewise, there was no appreciable difference in the first-order constant for efflux, indicating that loss of cytotoxicity resulting from deletion of N^3 in MTX could not be due to lack of ability to cross the cell membrane.

The IC_{50} value of (II.23) as an inhibitor of the growth of L1210 cells was found to be 44 μM, a value 8-fold higher than that of MTX and 54-fold higher than that of AMT [13]. This difference in potency was much less than would be predicted from the K_i values for DHFR inhibition in a cell-free assay (see above). However, binding of (II.23) to DHFR *in intact cells* proved to be more closely predictive of potency relative to MTX. For example, in an experiment in which L1210 cells were incubated with [^{3}H]MTX, the non-bound radioactive drug was allowed to efflux, an equimolar concentration of unlabelled MTX or (II.23) was added, and the displaced [^{3}H]MTX was allowed to efflux, it was found that (II.23) was only 2-fold less active than MTX in causing release of counts from the cells, even though there was a 7,000-fold difference in K_i against isolated DHFR. While the result of this experiment was difficult to interpret in the absence of data on either the ability of (II.23) to form polyglutamates or the affinity of such polyglutamates for DHFR (and other enzymes), the results were important in showing that the K_i from a purified enzyme assay should be used with caution to predict activity in an intact cell.

In view of the moderate activity of (II.23) *in vitro* and *in vivo*, the effect of additional pyridine ring substitution in this compound was of interest. Accordingly, electrophilic substitution reactions of (II.23) were carried out [14]. Bromination at 0 °C in a mixture of 6 M HCl and glacial acetic acid afforded (II.31) in 84% yield, and nitration at 0 °C in H_2SO_4 gave (II.32) in 90% yield. Curiously, there was no evidence, under these conditions, of any bromination or nitration of the phenyl ring. The site of electrophilic attack was confirmed to be in the pyridine ring by the disappearance of the NMR singlet at δ 6.07 and the lack of change in the A_2B_2 pattern of the phenyl ring protons.

The biological activities of compounds (II.31) and (II.32) were evaluated in several assay systems and compared with those of the unsubstituted 3-deaza analogue (II.23) [14]. The IC_{50} value of (II.23) as an inhibitor of pigeon liver DHFR was 0.063 μM, whereas the IC_{50} values for (II.31) and (II.32) were 2.5 and 38 μM, respectively. When each IC_{50} value was compared with that of MTX in the same experiment, the decrease in affinity was found to be 4.8-fold for (II.23), 190-fold for (II.31), and 1400-fold for (II.32). These results clearly demonstrated the harmful effect of bulky substitution at N^3 on binding to the avian enzyme. In assays of cell growth inhibition (H.Ep. 2 cells), (II.23) was 13-fold less potent than MTX, but (II.31) was 19,000-fold less potent, in accord with its low anti-DHFR activity. Similar low activity in comparison with MTX was demonstrated *in vivo* against L1210 murine leukaemia. While (II.23) had some activity (64% ILS at 100 mg/kg, qd × 9), (II.31) and (II.32) were completely inactive. This could be due to a difference in scheduling of doses, however, because the latter drugs were given only on days 2 and 6.



(II.31) X = Br
(II.32) X = NO_2

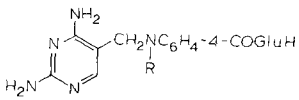
It is clear from the studies summarized above that structural modification of either the pyrimidine ring substituents or the pyrimidine ring itself has not generated useful MTX analogues. While the possibility cannot be ruled out that some still untried structural change, such as replacement of the 2-amino group by 2-hydrazino or 2-hydroxylamino, could give rise to an active DHFR inhibitor, the chances of achieving success by modifying this region are generally considered remote. The possibility that such modifications might give rise to new and novel inhibitors of folate pathway enzymes other than DHFR has not, however, been ruled out.

CHANGES IN REGION B

Several attempts have been made to modify the pyrazine ring in MTX with the aim of gaining insight into the role that this region plays in the biological activity of the molecule.

DELETION OF THE PYRAZINE RING

Interesting early examples of this type of modification are the MTX analogue (III.1) and the AMT analogue (III.2), in which the pyrazine moiety was entirely deleted [15]. The preparation of (III.1) began with 2,4-diamino-5-cyanopyrimidine, which was converted via the corresponding 5-formyl and 5-hydroxymethylpyrimidines into 2,4-diamino-5-bromomethylpyrimidine. Condensation of the bromomethyl compound with diethyl *N*-[4-(*N*-methylamino)benzoyl]-L-glutamate in DMF at room temperature, followed directly by saponification of the ester groups, afforded a 22% yield of (III.1). Similarly, reaction of the 5-bromomethylpyrimidine with *N*-(4-aminobenzoyl)-L-glutamic acid, without protection of the carboxyl groups, produced a 56% yield of (III.2). Alternatively, the 5-formylpyrimidine was condensed with dimethyl *N*-(4-aminobenzoyl)-L-glutamate to form a Schiff's base. Catalytic reduction in DMF solution, followed by alkaline hydrolysis led to (III.2), but the yield by this route was quite low (8%).

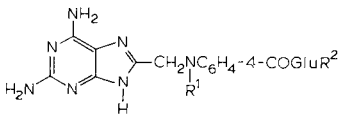


(III.1) R = Me
(III.2) R = H

The biological activity of (III.1) and (III.2) was evaluated in several *in vitro* and *in vivo* test systems. The AMT analogue (III.2) showed modest activity as a DHFR inhibitor, with an IC_{50} of 0.26 μM against partly purified murine enzyme. By comparison, the IC_{50} of MTX in the same assay was 0.02 μM. This less than 10-fold difference in potency was of interest because it suggested that the pyrazine ring might be less important than the pyrimidine ring as a determinant of enzyme binding. Curiously, when (III.2) was tested as an inhibitor of TS (from *E. coli*), it proved to have activity (IC_{50} = 6.5 μM) 15-fold greater than that of MTX (IC_{50} = 100 μM). Inhibition of *S. faecium* growth was also observed, but was lower than with (III.2) (IC_{50} = 2.1 nM) than with MTX (IC_{50} = 0.55 nM). Against a MTX-resistant *S. faecium* strain, cross resistance to (III.2) was essentially complete. Similarly there was complete cross-resistance between (III.2) and MTX in a strain of *S. faecium* resistant to homofolic acid. Marginal *in vivo* antitumour activity (25–50% ILS) was seen with both (III.1) and (III.2) against murine L1210 leukaemia, but only at high doses.

CONTRACTION OF THE PYRAZINE RING

The effect on biological activity of decreasing the size of the pyrazine ring in MTX and AMT by one carbon, as in the purine analogues (III.3) and (III.4) has also been investigated [16]. 2,4,5,6-Tetraaminopyrimidine was allowed to react successively with glycolic acid and thionyl chloride to obtain 2,6-diamino-8-chloromethylpurine, which on further reaction with diethyl *N*-[4(*N*-methylamino)benzoyl]-L-glutamate in the presence of KI gave the diester (III.5). Similar reaction with diethyl *N*-(4-aminobenzoyl)-L-glutamate afforded the diester (III.6). Ester hydrolysis in 1 M NaOH at room temperature led to the diacids (III.3) and (III.4), respectively. Compound (III.4) was also prepared by direct reaction of the chloromethylpurine with *N*-(4-aminobenzoyl)-L-glutamic acid, as well as by an alternate route involving reaction with 4-aminobenzoic acid followed by triacetylation, mixed anhydride coupling to dimethyl L-glutamate, and removal of amide and ester protecting groups. The MTX analogue (III.3) had the expected purine-like UV absorption spectrum, with maxima at 249 and 292 nm at pH 1 and a single maximum at 295 nm at pH 11. A slight hypsochromic shift was observed in the AMT analogue (III.4), suggesting some type of electronic interaction between the purine and 4-aminobenzoyl moieties which is influenced by the presence or absence of the N^{10}-methyl substituent. DHFR inhibition by (III.3) and (III.4) was assayed against chicken liver enzyme. Interestingly, the MTX analogue (III.3) (IC_{50} = 67 μM) was 4-fold more potent than the AMT analogue (III.4) (IC_{50} = 280 μM), but both analogues were over 10^3-fold less potent than their respective parent drug.

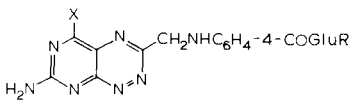


(III.3) R^1 = Me, R^2 = H
(III.4) R^1 = R^2 = H
(III.5) R^1 = Me, R^2 = Et
(III.6) R^1 = H, R^2 = Et

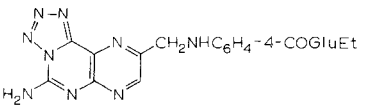
MODIFICATIONS AT POSITION 7

A less drastic structural modification of AMT was developed by Temple *et al.* [17, 18] in the form of the 7-aza analogue (III.7). Diethyl 4-(*N*-cyanomethylamino)benzoyl-L-glutamate, prepared from the 4-amino

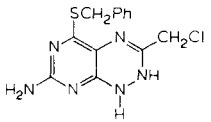
compound by *N*-cyanomethylation, was converted to an imino nitrile, and the latter was condensed *in situ* with 2,5-diamino-4-benzylthio-6-hydrazinopyrimidine to obtain, via spontaneous air oxidation of the initial 7,8-dihydro adduct, the pyrimido[5,4-*e*]-*as*-triazine (III.8) [18]. Treatment of (III.8) with NaN_3 in DMSO at 80 °C afforded a compound believed to be the diester (III.9) (30%), along with the fused tetrazole (III.11). Unfortunately, attempts to hydrolyze (III.9) met with failure. An improved route to (III.7) was therefore devised, which avoided the need for the hydrolysis step. In the modified synthesis [18], 2,5-diamino-4-benzylthio-6-hydrazinopyrimidine was condensed with ethyl orthochloroacetate, the resultant dihydro adduct (III.12) was oxidized to pyrimido[5,4-*e*]-*as*-triazine (III.13), which was condensed with *N*-(4-aminobenzoyl)-L-glutamic acid in the presence of KI to form the diacid III.10. Treatment with NaN_3 in DMSO at 90 °C converted (III.10) to (III.7).



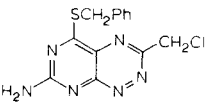
(III.7) X = NH_2, R = H
(III.8) X = SCH_2Ph, R = Et
(III.9) X = NH_2, R = Et
(III.10) X = SCH_2Ph, R = H



(III.11)



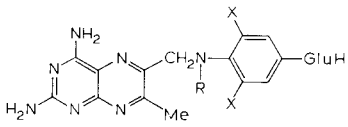
(III.12)



(III.13)

Biological testing of (III.7) revealed that replacement of the carbon at position 7 by nitrogen was highly detrimental [18]. The IC_{50} for inhibition of pigeon liver DHFR by (III.7) was 17 μM as compared with 0.002 μM for MTX, while against cultured KB cells (human epithelial carcinoma) the IC_{50} was $> 100\,\mu$M. Interestingly, however, the IC_{50} value of (III.7) against *S. faecium* was 0.027 μM, while that of 7-azafolic acid was 0.0015 μM [18, 19]. The basis of the growth inhibitory effect of (III.7) and 7-azafolic acid in the *S. faecium* system is not clear, since data were not obtained for the inhibition of either DHFR or TS from this organism. The authors suggested that activity might be due to intracellular reduction to di- or tetrahydro derivatives, but while this is conceivable for a 2-amino-4(3*H*)-oxo analogue, it seems rather improbable for a 2,4-diamine.

Further evidence that C-7 modification in classical antifolates is poorly tolerated has come from studies on the 7-methyl analogues (III.14)–(III.16) of AMT, MTX, and 3′,5′-dichloromethotrexate (DCM), respectively. Interest in these compounds lay in the possibility that they would prevent the catabolic action of hepatic aldehyde oxidase, which converts the 7-unsubstituted parent drugs (especially DCM) to less active 7-hydroxy derivatives. A synthesis of (III.14) and (III.15) was reported by Farquhar *et al.* [20], and a different synthesis leading to (III.15) and (III.16) was described by Rosowsky and Chen [21]. In the first study, 2,4,5,6-tetraaminopyrimidine was condensed with 1,4-dibromo-2,3-butanedione to give 2,4-diamino-6,7-bis(bromomethyl)-pteridine. The latter was treated with KI in warm aqueous THF, and the product, which was assumed to have been selectively reduced at position 7, was condensed with *N*-(4-aminobenzoyl)-L-glutamic acid in NaOAc buffer at pH 3 to obtain a product formulated as (III.14). The same procedure using *N*-[4-(*N*-methylamino)benzoyl]-L-glutamic acid afforded a compound claimed to be (III.15). The yields of purified products reported for the coupling reaction were quite high, 95% for (III.14) and 66% for (III.15). Supporting evidence for the structure of the products was obtained by alkaline $KMnO_4$ oxidation, which allegedly yielded 2,4-diamino-7-methylpteridine-6-carboxylic acid.



(III.14) R = X = H
(III.15) R = Me, X = H
(III.16) R = Me, X = Cl

In the synthesis of (III.15) and (III.16) by Rosowsky and Chen [21], which was an application of the regioselective Taylor pteridine synthesis [22–24], 2-amino-5-chloromethyl-3-cyano-6-methylpyrazine *N*-oxide was condensed with diethyl *N*-[4-(*N*-methylamino)benzoyl]-L-glutamate, the *N*-oxide group was removed with triethyl phosphite in DMF at 125 °C, and the resultant pyrazine amino nitrile was condensed with guanidine. Brief hydrolysis (15 min, 1 M NaOH in refluxing EtOH) led to (III.15), and chlorination of (III.15) at 5 °C afforded the 3′,5′-dichloro derivative (III.16). Ultraviolet absorption spectra of (III.15) and (III.16) in 0.1 M NaOH were consistent with those reported in the literature for MTX and DCM, except for small

bathchromic shifts reflecting the influence of the 7-Me substituent on the pteridine chromophore. Absorption maxima were observed at 261, 297, and 397 nm for (III.15) and at 262, 290 (infl) and 379 nm for (III.16). The data for (III.15) were somewhat different from those reported by Farquhar *et al.* [20].

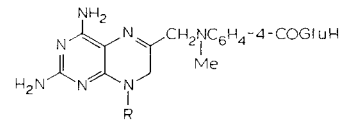
The compounds formulated as (III.14) and (III.15) [20] were tested as inhibitors of DHFR from rat liver and found to have IC_{50} values of 0.039 and 0.026 μM, respectively, as compared with 0.023 μM for MTX. Rosowsky and Chen [21], however, reported IC_{50}values of 7 μM for (III.15) and 4 μM for (III.16) against DHFR from murine L1210 leukaemia cells, as compared with 0.0015 μM for MTX and 0.001 μM for DCM. It thus appeared that, contrary to the earlier report, introduction of a 7-Me group in MTX and DCM produced a $> 10^3$-fold decrease in anti-DHFR activity. In order to rule out the possibility that the low activity of (III.15) and (III.16) was unique to the murine enzyme, the compounds were also tested as inhibitors of DHFR from *L. casei.* Once again, the 7-Me derivatives were less potent than the parent drugs by a factor of approximately 10^3. In growth inhibition assays in culture, (III.15) and (III.16) both had IC_{50} values at least 10-fold higher than those of MTX and DCM against *S. faecium* and also against mammalian tumour cells (P388 murine leukaemia, CCRF-CEM human lymphoblastic leukaemia). In an *in vivo* antitumour assay against L1210 leukaemia in mice, (III.15) failed to show significant activity ($< 25\%$ increase in survival) even at a dose of 160 mg/kg (q4d $\times$ 3); similarly, (III.16) was inactive at 320 mg/kg (q4d $\times$ 3). On the same q4d $\times$ 3 regimen, MTX and DCM gave significant therapeutic effects ($> 50\%$ increase in survival) at 15 and 120 mg/kg, respectively. In their *in vivo* antitumour assays, Farquhar *et al.* [20] similarly found (III.15) to be inactive, but reported marginal activity (30% increased in survival) with (III.14) when the drug was given qd $\times$ 9 at a dose of 40 mg/kg. It may be noted that an unsubstituted carbon at position 7 has been found to be important not only for the binding of 2,4-diamino compounds, but also for the binding of 2-amino-4(3*H*)-oxo compounds, as evidenced by the early finding of the Lederle group [25] that 7-methylfolic acid was inactive as either a growth substrate or growth inhibitor for *S. faecium.*

REDUCTION OF THE PYRAZINE RING AND MODIFICATION OF N^8

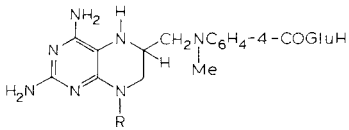
Reduction of the pyrazine ring to form dihydro and tetrahydro derivatives constitutes another interesting modification of ring B. This approach was stimulated initially by the findings of Kisliuk [26] and of Mead *et al.* [27] that reduced MTX and AMT derivatives possessed antitumour as well as antibacterial activity. Horwitz and Kisliuk [28] observed that reduction of MTX to

7,8-dihydroMTX (III.17) caused only a 2-fold decrease in anti-DHFR potency, while at the same time giving a 40-fold increase in anti-TS potency.

Although the affinity of (III.17) for TS was still far less than its affinity for DHFR, this early finding led to the concept that it might be possible to design compounds equally inhibitory to both enzymes. An attempt was made [29] to reduce this concept to practice by synthesizing a series of derivatives of (III.17) with alkyl substituents at N^8. It was reasoned that N^8 substitution would prevent reoxidation to MTX, which inevitably complicates the interpretation of biological data obtained with (III.17) itself. Alkylation of (III.17) was carried out by forming a lithium derivative *in situ* in DMSO solution and adding an alkyl or aralkyl halide. Yields were mostly in the 60–70% range. Compounds prepared in this manner were the N^8-substituted derivatives (III.18)–(III.25). Catalytic reduction of (III.18) furnished the 8-methyl-5,6,7,8-tetrahydro derivative, (III.26).



(III.17) R = H
(III.18) R = Me
(III.19) R = Et
(III.20) R = n-Bu
(III.21) R = n-Hx
(III.22) R = c-HxCH$_2$
(III.23) R = PhCH$_2$
(III.24) R = 3,4-Cl$_2$C$_6$H$_3$CH$_2$
(III.25) R = 1-C$_{10}$H$_7$CH$_2$



(III.26) R = Me
(III.27) R = H

Enzyme inhibition assays were carried out with (III.18)–(III.25) against DHFR and TS from *L. casei* [29]. Molar concentrations for 50% inhibition of DHFR ranged from 0.14 to 4.5 μM, while those for TS inhibition ranged from 4 to 43 μM. The most potent compound against both enzymes was (III.18) (R = Me), but (III.18) was not as potent as 7,8-dihydroMTX (III.17) itself, whose IC_{50} values against DHFR and TS were 0.02 and 1 μM, respectively. On the other hand, the compounds for which the IC_{50}(DHFR)/IC_{50}(TS) ratio was closest to 1.0 were the 8-aralkyl derivatives (III.24) and (III.25). For example, (III.24) had IC_{50} values of 3.6 and 13 μM against against DHFR and TS, respectively, and therefore had an IC_{50}(DHFR)/IC_{50}(TS) ratio of only 3.6. Comparison of the 7,8-dihydro and 5,6,7,8-tetrahydro analogues (III.18) and (III.26) showed the former to be slightly more potent against both enzymes, just as (III.17) was more potent than 5,6,7,8-tetrahydroMTX (III.27).

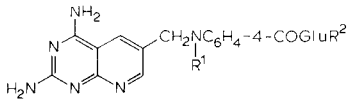
An indication of the effect of 8-alkyl and 8-aralkyl substitution on *in vitro* antibacterial and antitumour activity was obtained against *L. casei* and against CCRF-CEM human leukaemic lymphoblasts in culture [29]. The most active compound against *L. casei* was (III.18) (IC_{50} = 0.1 nM), but its potency was 5-fold lower than that of (III.17) and 2.5-fold lower than that of MTX. However the IC_{50} values of (III.18)–(III.25) against human cells were only in the 0.1–1.0 mM range, and potency was again decreased in comparison with (III.17) and MTX. Antitumour activity *in vivo* against L1210 leukaemia in mice was negligible, and consequently work in this series was discontinued.

REPLACEMENT OF C BY N IN THE PYRAZINE RING

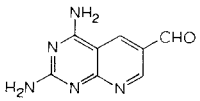
5-Deaza compounds

Chemical synthesis

Elegant syntheses of the 5-deaza analogues (III.28) and (III.29) have been developed independently by three groups [30–34]. One synthesis, described by Temple *et al.* [30] began with 2,4,6-triaminopyrimidine, which on reaction with bromoacetic acid and the triformylmethane equivalent $CH[CH{=}NMe_2]_3Cl_3$ afforded a 33% yield of 2,4-diaminopyrido[2,3-*d*]pyrimidine-6-carboxaldehyde (III.32). Reductive condensation of (III.32) with diethyl *N*-(4-aminobenzoyl)-L-glutamate in the presence of Raney Ni produced the diester (III.30), and hydrolysis of (III.30) with 1 M NaOH in DMSO at room temperature gave (III.28). Treatment of (III.28) with aqueous formaldehyde and $NaCNBH_3$ at pH 6.4 led to the MTX analogue (III.29) in high yield. This procedure appears to be a general means of converting AMT analogues to MTX analogues.



(III.28) $R^1 = R^2 = H$
(III.29) R^1 = Me, R^2 = H
(III.30) R^1 = H, R^2 = Et
(III.31) R^1 = H, R^2 = Me

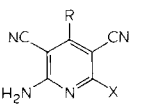


(III.32)

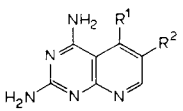
In the Taylor synthesis of (III.28) [31], aldehyde (III.32) was obtained by reaction of 2-amino-3-cyano-5-(dimethoxymethyl)pyridine and guanidine, followed by cleavage of the acetal with aqueous formic acid. The pyridine aminonitrile was elaborated in a sequence of high-yield steps from 3-cyano-5-methylpyridine-2-thione. These steps consisted of the following (yields in

parentheses): *S*-arylation with 1-fluoro-4-nitrobenzene (97%); bromination of the 5-Me group with *N*-bromosuccinimide and conversion to a 5-(1-pyridinium)methyl derivative (55%); formation of a nitrone via a Krohnke reaction with 4-nitroso-*N*,*N*-dimethylaniline (95%); cleavage of the nitrone to 2-(4-nitrophenylthio)-3-cyanopyridine-5-carboxaldehyde (90%); acetalization with acidic methanol (88%); and replacement of the arylthio group by an amino group with liquid NH_3 in the presence of $CuBr_2$ (91%). Reaction of (III.32) with dimethyl *N*-(4-aminobenzoyl)-L-glutamate in the presence of $NaCNBH_3$, followed by hydrolysis of the resultant diester (III.31), gave a 28% yield (two steps) of the diacid (III.28).

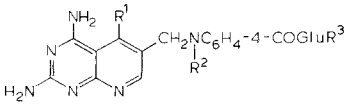
Recently an improved synthesis of (III.28) and (III.29) that also allowed the 5-Me congeners (III.41) and (III.43) to be prepared was described by Piper *et al.* [32a]. Essentially the same synthetic approach to (III.28) was reported independently by Elslager and Davoll [32b]. Malononitrile was condensed with ethyl orthoformate or ethyl orthoacetate in pyridine to give intermediates from which were formed, on treatment with acid, the dinitriles (III.33) and (III.34). Reductive dehalogenation of (III.33) and (III.34) (H_2-Pd-$BaCO_3$) gave the dinitriles (III.35) and (III.36), from which the pyrido[2,3-*d*]pyrimidine-6-carbonitriles (III.37) (95%) and (III.38) (55%) were prepared by condensation with guanidine. The lower yield of (III.38) in comparison with (III.37) presumably reflected steric hindrance to ring closure. Reductive coupling of (III.37) and (III.38) to diethyl *N*-(4-aminobenzoyl)-L-glutamate gave the diesters (III.30) and (III.44). Hydrolysis of the diesters gave (III.28) and (III.41), respectively. Treatment of (III.41) with formaldehyde in the presence of excess $NaCNBH_3$ led to the 5,10-dimethyl derivative (III.42).



(III.33) R = H, X = Cl
(III.34) R = Me, X = Cl
(III.35) R = X = H
(III.36) R = Me, X = H



(III.37) R^1 = H, R^2 = CN
(III.38) R^1 = Me, R^2 = CN
(III.39) R^1 = Me, R^2 = CHO
(III.40) R^1 = Me, R^2 = CH_2Br

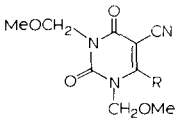


(III.41) R^1 = Me, R^2 = R^3 = H
(III.42) R^1 = R^2 = Me, R^3 = H
(III.43) R^1 = Me, R^2 = Et, R^3 = H
(III.44) R^1 = R^2 = H, R^3 = Et
(III.45) R^1 = R^2 = Me, R^3 = Et
(III.46) R^1 = Me, R^2 = R^3 = Et

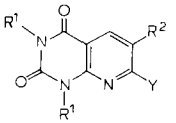
Because the yields of diesters (III.30) and (III.44) from nitriles (III.35) and (III.36) were only 15%, an alternative route was sought [32a]. Nitrile (III.38) was converted to aldehyde (III.39) by treatment with formic acid and Raney Ni, and the aldehyde was reduced with $NaBH_4$ and converted directly to 2,4-diamino-6-bromomethyl-5-methylpyrido[2,3-*d*]-pyrimidine (III.40) by bromination with dibromotriphenylphosphorane in *N*,*N*-dimethylacetamide (DMA). Coupling of (III.40) to dimethyl *N*-[4-(*N*-methylamino)benzoyl]-L-glutamate and diethyl *N*-[4-(*N*-ethylamino)benzoyl]-L-glutamate afforded the diesters (III.45) and (III.46), respectively. While the yield of (III.45) from (III.40) was 27%, that of (III.46) was unfortunately only 8%. Hydrolysis of the diesters gave the acids (III.42) and (III.43).

A third route to the 5-deaza analogues of AMT and folic acid, incorporating several novel features, was developed recently [33]. The starting point for assembly of the 5-deazapteridine moiety was 5-cyano-1,3-bis(methoxymethyl)uracil (III.47), which was prepared either from 5-cyanouracil by alkylation with chloromethyl methyl ether or, preferably, from 1,3-bis(methoxymethyl)uracil by bromination followed by treatment with NaCN. Condensation of (III.47) with malononitrile in the presence of NaOEt afforded the amino nitrile, (III.49), which on diazotization in concentrated HCl was converted to the chloro nitrile (III.51) (71% yield) along with a small amount (8%) of the corresponding hydroxy nitrile. Hydrogenolysis of (III.51) in the presence of 10% Pd-C in dioxane led to the dehalogenated product (III.53) (88%). Reduction of (III.53) over Raney Ni in Ac_2O-AcOH followed by *N*-nitrosation with $NaNO_2$ in Ac_2O-AcOH and heating in Ac_2O resulted in the formation of the protected 6-acetoxymethyl derivative (III.55) in 67% overall yield (three steps). Deprotection of (III.55) with BCl_3 and reacetylation with Ac_2O-C_5H_5N afforded the alcohol (III.57) (83%) and ester (III.59) (80%), respectively. The next step in the sequence, and the key to its success, was the direct amination of (III.59), which was accomplished in 74% yield by heating the 2,4-dioxo compound in a mixture of liquid ammonia, hexamethyldisilazane, and p-TsOH in an autoclave at 160 °C for 5 days. The resulting alcohol, (III.61), was converted to bromide (III.62) with dry HBr in dioxane, and the latter was condensed directly with excess diethyl *N*-(4-aminobenzoyl)-L-glutamate in DMA (3 days at room temperature) to obtain the diester (III.30) (36%). Alkaline hydrolysis (1 M NaOH in MeOH) afforded the diacid (III.28) (45%). The same sequence starting from 1,3-bis(methoxymethyl)-5-cyano-6-methyluracil (III.48) led to the 5-methyl-5-deazapteridines (III.50,52,54,56,58,60) and ultimately the diester (III.78) and diacid (III.79). Yields for the 5-unsubstituted and 5-methyl series were comparable. However, condensation of the 5-cyano-6-methyluracil derivative with malono-

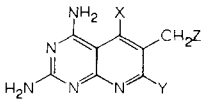
nitrile required heating to 60 °C and gave a yield of only 45%, whereas the 5-cyanouracil derivative had reacted readily at room temperature and given a yield of 85%.



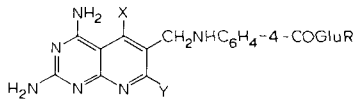
(III.47) R = H
(III.48) R = Me



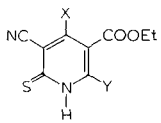
(III.49), (III.50) R^1 = $MeOCH_2$, R^2 = CN, X = H, Me; Y = NH_2
(III.51), (III.52) R^1 = $MeOCH_2$, R^2 = CN, X = H, Me; Y = Cl
(III.53), (III.54) R^1 = $MeOCH_2$, R^2 = CN, X = H, Me; Y = H
(III.55), (III.56) R^1 = $MeOCH_2$, R^2 = CH_2OAc, X = H, Me; Y = H
(III.57), (III.58) X = H, Me, R^1 = Y = H, R^2 = CH_2OH
(III.59), (III.60) X = H, Me, R^1 = Y = H, R^2 = CH_2OAc



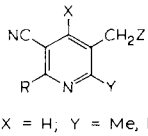
III.61), (III.62) X = Y = H; Z = OH, Br
III.63), (III.64) X = H; Y = Me, Ph; Z = OCH_2OMe
III.65), (III.66) X = H; Y = Me, Ph; Z = OH
III.67), (III.68) X = H; Y = Me, Ph; Z = Br
III.69 – III.71) X = Y = Me; Z = OCH_2OMe, OH, Br
III.72 – III.74) X = Me; Y = Ph; Z = OCH_2OMe, OH, Br
III.75 – III.77) X = Ph, Y = Me; Z = OCH_2OMe, OH, Br



(III.78), (III.79) X = Me; Y = H; R = Et, H
(III.80), (III.81) X = H; Y = Me; R = Et, H
(III.82), (III.83) X = H; Y = CH_2OEt, Ph; R = Et
(III.84), (III.85) X = H; Y = CH_2OEt, Ph; R = H
(III.86), (III.87) X = Y = Me; R = Et, H
(III.88), (III.89) X = Me; Y = Ph; R = Et, H
(III.90), (III.91) X = Ph; Y = Me; R = Et, H



(III.92) X = H, Y = Me
(III.93) X = H, Y = CH_2OEt
(III.94) X = H, Y = Ph
(III.95) X = Y = Me
(III.96) X = Me, Y = Ph
(III.97) X = Ph, Y = Me

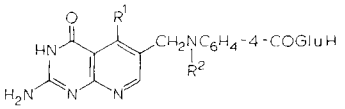


(III.98), (III.99) X = H; Y = Me, Ph; Z = OH; R = SMe
(III.100), (III.101) X = H; Y = Me, Ph; Z = OCH_2OMe; R = SMe
(III.102), (III.103) X = H, Y = Me, Ph; Z = OCH_2OMe; R = SO_2Me
(III.104) X = Y = Me, Z = OH; R = SMe
(III.105), (III.106) X = Y = Me; Z = OCH_2OMe; R = SMe, SO_2Me
(III.107), (IV.108) X = Me; Y = Ph; Z = OCH_2OMe; Z = SMe, SO_2Me
(III.109), (III.110) X = Ph; Y = Me; Z = OCH_2OMe; Z = SMe, SO_2Me

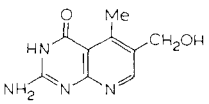
In a follow-up study [34] it was discovered that the condensation of cyanothioacetamide with ethyl 2-ethoxymethylene-3-oxobutanoate in the presence of piperidine in fact gave a 41% yield of 3-cyano-5-ethoxycarbonyl-6-methylpyridine-2(1*H*)-thione (III.92), and *not the isomeric 4-methyl derivative* as previously thought [32]. Methylation of (III.92) (MeI-K_2CO_3-DMF) followed by reduction ($LiAlH_4$-Et_2O), methoxymethylation, and oxidation with *m*-chlorobenzoic acid led to the alcohol (III.98) (50%), the ether (III.100)

(84%) and the methylsulphone (III.102) (75%). On condensation with guanidine carbonate in diphenyl ether at 180–185 °C, (III.102) was converted to (III.63) (67%), which on reaction first with HCl in MeOH and then with HBr in dioxan gave the alcohol (III.65) and the bromide (III.67). Condensation of (III.67) with diethyl *N*-(4-aminobenzoyl)-L-glutamate followed by alkaline hydrolysis afforded the diester (III.80) and diacid (III.81). Use of ethyl 4-ethoxy-2-ethoxymethylene-3-oxobutanoate, ethyl 2-ethoxymethylene-3-oxo-4-phenylpropanoate, and ethyl 2-acetyl-3-oxobutanoate in place of ethyl 2-ethoxymethylene-3-oxobutanoate in this sequence afforded the pyridines (III.93)–(III.95) and deazapteridines (III.64), (III.66), and (III.68)–(III.71). Replacement of 2-ethoxymethylene-3-oxobutanoate with ethyl 2-benzoyl-3-oxobutanoate yielded a mixture of the pyridines (III.96) (major isomer) and (III.97) (minor isomer), which were separated by chromatography and individually converted to the intermediate pyridines (III.107)–(III.110), 5-deazapteridines (III.72)–(III.77), and 5-deazaAMT analogues (III.88)–(III.91). The 7-methyl-5-deazapteridines could be distinguished from the 5-methyl-5-deazapteridines by their ^1H and ^{13}C resonance spectra. For example, the C-5 proton in alcohol (III.65) gave rise to a singlet at δ 8.54 whereas the C-7 proton in alcohol (III.61) showed a singlet at δ 8.64, as would be expected from its closer proximity to the ring nitrogen.

5-Deazafolic acid (III.111), 5-methyl-5-deazafolic acid (III.112), and 5,10-dimethyl-5-deazafolic acid (III.113) have been prepared in 80–85% yield by heating the corresponding 2,4-diamines (III.28), (III.41) and (III.42) (see above) for 4.5 h in 1 M NaOH at reflux [32a]. In an alternative route to (III.111) [33], hydrolysis of the 4-amino group in (III.58) gave the 2-amino-4(3*H*)-oxo compound (III.114) in 82% yield. Bromination (HBr-dioxan) followed by coupling to diethyl *N*-(4-aminobenzoyl)-L-glutamate and ester hydrolysis then gave diacid (III.112).



(III.111) $R^1 = R^2 = R^3 = H$
(III.112) $R^1 = Me$, $R^2 = R^3 = H$
(III.113) $R^1 = R^2 = Me$, $R^3 = H$



(III.114)

Biological activity

Data have been reported on the biological activity of AMT and MTX analogues (III.28) and (III.29), as well as on the activity of the 5-Me congeners

(III.41) and (III.42) [32a]. Compound (III.28) was a potent inhibitor of beef liver DHFR, with an IC_{50} value of 7.1 nM which was close to that of AMT (IC_{50} = 5 nM). The ability of (III.28) to inhibit DHFR from L1210 cells and from chicken liver was also similar to that of AMT, as measured by the K_i, but was roughly 200-fold lower against DHFR from *E. coli*. The kinetics of the interaction of (III.28) with both avian and bacterial enzyme exemplified slow tight-binding inhibition, and there was no evidence of isomerization of the enzyme-inhibitor complex [35]. AMT also behaved in this manner with the chicken liver enzyme but not with the *E. coli* enzyme, for which there was, instead, kinetic evidence of isomerization after the complex was formed. Negligible inhibition of TS by (III.28) ($IC_{50} > 10\ \mu M$) was observed. Introduction of a 5-Me substituent to give (III.41) led to a small decrease in the K_i for inhibition of DHFR from L1210 cells, from 3.7 nM to 2.9 nM, suggesting that a lipophilic group at position 5 may be favourable for binding to the active site. In assays against DHFR from L1210 cells [34], the introduction of substituents at the 7-position of 5-deazaAMT (i.e., in compounds (III.81), (III.84), (III.85), (III.89) and (III.91) was highly detrimental to binding. In accord with these findings, the 7-substituted compounds were much less potent than either MTX or an authentic sample 5-methyl-5-deazaAMT (III.79) against L1210 cells in culture. In fact, it was the low biological activity of (III.81) which provided the first indication that the original formulation [33] of this compound as the 5-methyl derivative was incorrect. That 7-substitution interferes markedly with the binding of pteridine antifolates had been demonstrated previously by other investigators [20, 21].

Results of DHFR inhibition studies with the MTX analogue (III.29) have likewise been reported [35]. The K_i values for inhibition of the L1210, *E. coli*, and chicken liver enzyme by (III.29) are all slightly lower than those of MTX, and the kinetics of binding of both drugs to the bacterial and avian enzyme are similar, reflecting slow, tight binding followed by isomerization of the enzyme-inhibitor complex. The detailed kinetic analysis of inhibition of DHFR by (III.28) and (III.29) seemed to suggest that a 10-Me group may qualitatively alter the binding of the 5-deaza analogues but not of the parent compounds. The availability of the 5-deaza analogues has thus made it possible to uncover remarkable subtleties in the kinetics of DHFR inhibition that might otherwise have been overlooked. Detailed kinetic data for the interaction of the 5,10-dimethyl analogue (III.42) and the 5-methyl-10-ethyl analogue (III.43) with DHFR have not yet been published. However, both compounds are somewhat better inhibitors of the L1210 enzyme than MTX [32a], suggesting again that a lipophilic 5-substituent may favour binding (see above).

Data on the ability of the AMT analogue (III.28) to inhibit the growth of cultured cells have been obtained with L1210 cells and with a melanoma line (G361). The IC_{50} value against L1210 cells was reported to be 38 nM versus 5.9 nM for MTX by one laboratory [33] and 0.72 nM versus 2.6 nM for MTX by another [32a]. There is thus some disagreement as to whether (III.28) is more potent or less potent than MTX. It should be noted, however, that in the assays where (III.28) was found to be more potent than MTX, the cells were exposed to drug for 72 h rather than 48 h, which might have allowed for more extensive polyglutamylation. In addition small variations in the composition of the growth medium used in the two laboratories might have affected the assay results. When the IC_{50} values of (III.28) and (III.29) against L1210 cells were compared, there was almost the same 4-fold decrease in potency as was observed between AMT and MTX. Interestingly, 5-Me substitution always produced a marked increase in potency, with (III.41)–(III.43) all giving IC_{50} values in the 0.1–0.3 nM range as compared with 0.72 nM for AMT. The increased growth inhibitory effect of these 5-alkyl-5-deaza derivatives was consistent with their enhanced ability to inhibit DHFR, and it was clear that they represented a highly potent group of antifolates in comparison with the 1-deaza and 3-deaza series.

An important question that had to be addressed with regard to the high cytotoxicity of 5-alkyl-5-deaza analogues of AMT and MTX was whether this is due only to tight binding to DHFR, or whether the compounds also have an increased ability to be transported into cells and converted to non-effluxing polyglutamates. The transport K_m values for unidirectional influx were measured for (III.28), (III.29), and (III.41)–(III.43), and compared with those for AMT and MTX [32a]. The K_m values for (III.28), (III.41), and AMT were identical (1.1–1.2 μM), indicating that the increased potency of (III.41) is not due merely to better cell membrane penetration. The K_m values for (III.29), (III.42), (III.43) and MTX were similar (3.2–4.0 μM) and somewhat higher than those for the N^{10}-unsubstituted analogues, indicating that, in the 5-deazapteridine series, as in the pteridine series, transport across the cell membrane depends to some degree on the presence or absence of a 10-alkyl group. Efflux rates from drug-loaded cells were also measured, and were found to be similar (k = 0.26–0.33 min^{-1}) regardless of structure. Polyglutamylation rates were determined for the compounds, but here the 5-deaza analogues proved inferior to the parent drug and the presence of a 5-Me substituent had no enhancing effect. It was noteworthy, on the other hand, that the AMT analogues (III.28) and (III.41) (80–100 pmol/min per g dry wt.) were polyglutamylated more rapidly than the MTX analogues (III.29), (III.42) and (III.43) (40–60 pmol/min per g dry wt.). From these data it would appear that

the major reason for the high toxicity of the 5-deaza analogues to cultured L1210 cells is their ability to inhibit DHFR.

The *in vivo* antitumour activity of the 5-deaza analogues of AMT and MTX was determined in mice [32a]. Against L1210 murine leukaemia on a qd × 9 schedule, (III.28) gave a 100% increase in lifespan (ILS) at 1 mg/kg and was toxic at 8 mg/kg. Against P388 murine leukaemia on a qd × 5 schedule, (III.28) gave a 46% ILS at 0.5 mg/kg, while the 5-Me congener (III.41) gave a 51% ILS at the same dose. In the MTX analogue series, again on a qd × 5 schedule, (III.29) gave a 66% ILS at 4 mg/kg, while (III.42) gave an 82% ILS at 1 mg/kg. The ILS of MTX was 82% at 4 mg/kg. Thus, both groups of 5-deaza compounds produced similar therapeutic effects but unfortunately were not superior to MTX. The addition of a 5-Me group somewhat decreased the therapeutic dose of (III.42) in comparison with (III.29), but not of (III.41) in comparison with (III.28). All the compounds were tested also against a P388 subline resistant to MTX, but essentially complete cross-resistance was observed. The diethyl ester (III.30) was likewise tested against the P388 and P388/MTX tumours, but gave the same results as the parent acid, presumably because of rapid cleavage of the ester groups by nonspecific serum esterases, whose levels in mouse serum are high.

In antibacterial assays against *S. faecium* and *L. casei*, 5-deazafolic acid (III.111) proved to be only 3- to 5-fold less potent than MTX, with IC_{50} values in the 0.1–0.2 nM range [32a]. At least some of the activity of the compound was probably due to DHFR inhibition, since the IC_{50} against purified *L. casei* enzyme was 0.07 μM as compared with 0.016 μM for MTX. The binding affinity of (III.111) was 240-fold lower for TS (IC_{50} = 17 μM) than for DHFR, suggesting that TS was not likely to be an important locus of action. Resistance to (III.111) in 3,000-fold MTX-resistant *S. faecium* and > 500,000-fold MTX resistant *L. casei* was found to be 49- and 3,000-fold, respectively. Thus the MTX-resistant organisms had relatively low cross-resistance to the 5-deazafolate analogue, suggesting that this compound might be taken up better, or might be toxic via some mechanism independent of DHFR inhibition. Reversal studies (e.g., with hypoxanthine to reveal inhibition of *de novo* purine synthesis) were not reported, but would be of interest.

In assays of DHFR and TS inhibition as well as *S. faecium* and *L. casei* growth inhibition [32a], (III.112) and (III.113) proved considerably less active than (III.111). In the DHFR assay, potency was decreased 140-fold by 5-Me substitution and 400-fold by 5,10-Me_2 substitution. Potency against *S. faecium* relative to (III.111) was decreased 4,200-fold in (III.112) and 330-fold in (III.113), whereas potency against *L. casei* was decreased 11-fold in (III.112) and 85-fold in (III.113). It thus appeared that modification of N^5

and N^{10} in (III.111) was quite detrimental to antibacterial activity, although the effect was species-related.

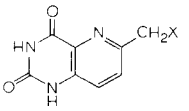
Compounds (III.111)–(III.113) were also tested against L1210 cells in culture [32a], but were found to have too little growth inhibitory activity in comparison with AMT and MTX to warrant further mechanistic and therapeutic evaluation. It was of interest, however, that the 5-Me analogue (III.112) (IC_{50} = 0.29 μM) was approximately 10-times more potent than (III.111) (IC_{50} = 3.2 μM) or (III.113) (IC_{50} = 2.4 μM) against these mammalian cells, even though it was a poorer inhibitor of DHFR from *L. casei*.

8-Deaza compounds

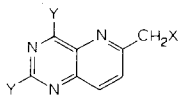
2,4-Diamino derivatives

Replacement of carbon by nitrogen at position 8 of the pteridine ring in MTX and AMT has been accomplished by Srinivasan and Broom [36]. Starting from 6-bromomethylpyrido[3,2-*d*]pyrimidine-2,4(1*H*,3*H*)-dione (III.115), the *N*-oxide was prepared by *m*-chloroperbenzoic acid oxidation and subjected to Polonovski rearrangement (Ac_2O-AcOH) to obtain the 6-acetoxymethyl derivative (III.116). Treatment of (III.116) with $POCl_3$ afforded the 2,4-dichloro derivative (III.117), which on being heated at 160–170 °C in liquid ammonia in a pressure vessel was converted to the 2,4-diamino-6-hydroxymethyl compound, (III.118). The latter was treated with PBr_3 in dry THF to form the corresponding bromide (III.119), which was coupled directly to diethyl *N*-(4-aminobenzoyl)-L-glutamate or diethyl *N*-[4-(*N*-methylamino)benzoyl]-L-glutamate in DMA to obtain the esters (III.120) (53%) and (III.121) (61%), respectively. Hydrolysis of the esters led to the AMT and MTX analogues (III.122) (68%) and (III.123) (72%). The NMR spectrum of (III.122), taken in d_6-DMSO-D_2O, showed the CH_2N protons as a singlet at δ 4.6, the phenyl protons as a pair of doublets (A_2B_2 pattern) at δ 6.9 and 7.9, and the C-7 and C-8 protons as an unresolved single peak at δ 7.8. The spectrum of (III.123), in the same solvent, showed the CH_2N protons as a singlet at δ 3.27, the phenyl protons as a pair of doublets at δ 6.9 and 7.9, and the C-7 and C-8 protons as a pair of singlets at δ 7.58 and 7.85. The upfield displacement of the chemical shift for the CH_2N protons by 1.3 ppm in the MTX analogue (III.121) relative to the AMT analogue (III.120) was a notable feature, as was the fact that in (III.121) the C-7 and C-8 proton resonances were resolved, whereas in (III.120) they were not. These effects are consistent with the view that the steric configuration in the bridge region is different in compounds with an alkyl substituent at position 10 from that in compounds lacking such a substituent. The UV spectra of

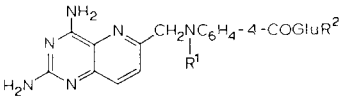
(III.120) [λ_{max} (pH 13) 278 nm (ε 25,000), 355 (8,000); λ_{max} (pH 7) 278 nm (ε 22,300), 355 (8,000); λ_{max} (pH 1) 318 nm (ε 10,000)] and (III.121) [λ_{max} (pH 13) 278 nm (ε 19,500), 307 (25,500); λ_{max} (pH 7) 275 nm (ε 16,000), 305 (20,400); λ_{max} (pH 1) 318 nm (ε 11,000), 333 (7,700)] revealed striking differences between these compounds with regard to the position and intensity of the long-wavelength bands at both acidic and alkaline pH. Since absorbance in this region is presumably due to the 2,4-diaminopyrido[3,2-*d*]-pyrimidine chromophore, it is not clear why substitution on N^{10} has such marked long range effect.



(III.115) X = Br
(III.116) X = OAc



(III.117) X = OAc, Y = Cl
(III.118) X = OH, Y = NH_2
(III.119) X = Br, Y = NH_2



(III.120) R^1 = H, R^2 = Et
(III.121) R^1 = Me, R^2 = Et
(III.122) R^1 = R^2 = H
(III.123) R^1 = Me, R^2 = H

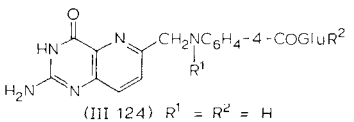
Recently reported biological studies [37] revealed that 8-deazaAMT (III.122) and 8-deazaMTX (III.123) possess high antitumour activity *in vitro* and *in vivo*. In assays against CEM hyman lymphoblastic leukaemia cells in culture, (III.122) and (III.123) had IC_{50} values of 3.5 and 6.7 nM, respectively. The corresponding IC_{50} values for AMT and MTX were 5.2 and 12 nM. Thus, N-for-C substitution at position 8 resulted in a 1.5- to 2-fold increase in cytotoxicity. This appeared to reflect, at least in part, an increase in cellular accumulation. The K_m for unidirectional influx was 3.4 μM for (III.122) versus 5.1 μM for AMT, and 1.1 μM for (III.123) versus 4.0 μM for MTX. Plateau levels of both 8-deaza compounds after several hours were higher than those of the corresponding pteridines, and (III.123) accumulated to a higher level than did (III.122). The same increase in accumulation occurred with AMT in comparison with MTX. Interestingly, there was a difference in polyglutamylation between (III.123) and MTX in CEM cell. While the major species from MTX after 24 h of incubation in 10 μM drug was the triglutamate, with (III.123) the principal metabolite was the diglutamate. Furthermore, while MTX formed di- to pentaglutamates, (III.123) did not proceed beyond the triglutamate stage.

8-DeazaAMT (III.122) was tested for *in vivo* antitumour activity against

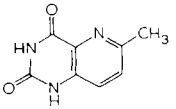
P388 and L1210 leukaemia in mice [37]. Against the P388 tumour on a q3d × 2 schedule, an optimal dose of 6.7 mg/kg afforded a 180% ILS. With AMT in this system there was a similar optimal dose of 10 mg/kg, but the ILS was only 80%. The L1210 tumour appeared to be more sensitive; in this case the optimal dose was only 1.6 mg/kg, which gave a 130% ILS. An aspect of (III.122) and (III.123) which is worth noting is that replacement of the nitrogen at position 8 by carbon prevents 7-hydroxylation by hepatic aldehyde oxidase, a pharmacologically important metabolic detoxification mechanism with MTX [38].

*2-Amino-4(3*H*)-oxo derivatives*

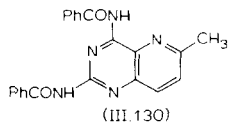
The earliest reported synthesis of 8-deazafolic acid (III.124) was one by DeGraw *et al.* [37a], based on the prior work of Oakes, Rydon and Undheim [37b]. 6-Methylpyrido[3,2-*d*]pyrimidine-2,4(1*H*,3*H*)-dione (III.129) was elaborated in several steps to 2,4-dibenzamido-6-methylpyrido[3,2-*d*]pyrimidine (III.130), which on condensation with ethyl 4-aminobenzoate and alkaline hydrolysis afforded 8-deazapteroic acid (III.131). The 2-amino and N^{10} nitrogens in (III.131) were protected by acetylation and trifluoroacetylation, respectively, and the resulting product (III.132) was coupled to diethyl L-glutamate by the mixed carboxylic-carbonic anhydride method to form the diester (III.133). Alkaline hydrolysis simultaneously removed the ester and amide blocking groups, yielding (III.124) in 17% yield (coupling and deprotection). The same product was also formed in better yield by using α-benzyl-γ-glutamyl Merrifield resin instead of diethyl L-glutamate. UV spectral data for (III.124) [λ_{max} (pH 1) 252 nm (ε 14,950), 305 (10,300); λ_{max} (pH 13) 285 nm (ε 22,000)] appeared to be consistent with its assigned structure.



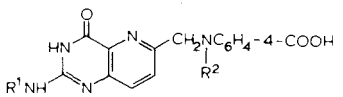
(III.124) $R^1 = R^2 = H$
(III.125) R^1 = Me, R^2 = H
(III.126) R^1 = H, R^2 = Et
(III.127) R^1 = Me, R^2 = Et
(III.128) R^1 = NO, R^2 = Et



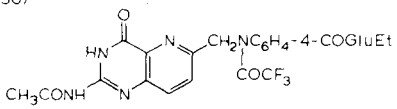
(III.129)



(III.130)

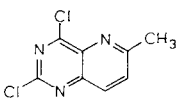


(III.131) $R^1 = R^2 = H$
(III.132) $R^1 = CH_3CO$, $R^2 = CF_3CO$

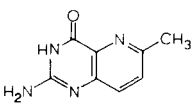


(III.133)

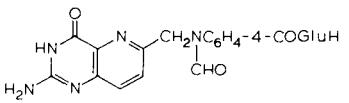
In a variant of the above approach, Temple *et al.* [37c] converted (III.129) successively to the dichloride (III.134) (72%) and the 2-amino-4(3*H*)-oxo derivative (III.135) (75%). Treatment of (III.135) with acetic anhydride and sulphuric acid followed directly by bromination at 95 °C in acetic acid in the presence of sodium acetate, condensation with *N*-(4-aminobenzoyl)-L-glutamic acid, and alkaline hydrolysis afforded a low yield of (III.124), whose UV spectral properties [λ_{max} (pH 1) 250 nm (ε 15,100), 298 (19,400); λ_{max} (pH 13) 283 nm (ε 25,000), 292sh (24,000)] were similar to, though not identical with, those reported by De Graw *et al.* [37a]. Platinum-catalyzed hydrogenation of (III.124) in formic acid gave only the N^{10}-formyl derivative (III.136) [λ_{max} (pH1) 252 (ε 27,900), 307 (6,200), 321sh (4,900); λ_{max} (pH 13) 244 nm (ε 35,300), 270sh (21,500), 335 (6,100)], whereas similar reduction in trifluoroacetic acid afforded a mixture of (III.137) (13%), 2-amino-6-methyl-5,6,7,8-tetrahydropyrido[3,2-*d*]pyrimidin-4(3*H*)-one (20%), and *N*-(4-aminobenzoyl)-L-glutamic acid (38%). Compound (III.137) was unstable and could only be isolated as the N^{10}-formyl-5,6,7,8-tetrahydro derivative (III.138) [λ_{max} (pH 13) 253 nm (ε 21,200), 310 sh (5,300)]. When (III.136) was catalytically reduced in the presence of trifluoroacetic acid the product was found to be the (III.139) [λ_{max} (pH 13) 220 nm (ε 15,800), 331 (28,500)], which on treatment with base underwent ring opening to (III.138).



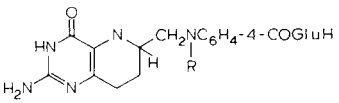
(III.134)



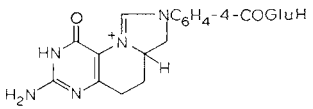
(III.135)



(III.136)



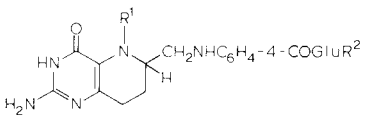
(III.137) R = H
(III.138) R = CHO



(III.139)

8-Deazafolic acid (III.124) was also synthesized [37] as an intermediate to the novel multisubstrate analogue (III.140) [39], which was designed with the intent of mimicking the species thought to be generated during the TS-catalyzed transfer of a carbon from 5,10-methylenetetrahydrofolate to deoxyuridylate to form thymidylate. In the course of this work, 10-methyl-8-deazafolic acid (III.125) was likewise prepared [37]. Treatment of 2-amino-6-(hydroxymethyl)pyrido[3,2-*d*]pyrimidin-4(3*H*)-one with PBr_3 in THF followed directly by reaction with diethyl *N*-(4-aminobenzoyl)-L-glutamate or diethyl *N*-[4-(*N*-

methylamino)benzoyl]-L-glutamate afforded the diesters (III.126) (68%) and (III.127) (58%), respectively. Alkaline hydrolysis then gave (III.124) (65%) and the 10-methyl analogue (III.125) (58%). The NMR spectrum of (III.124), taken in d_6-DMSO-D_2O, showed the CH_2N protons as a singlet at δ 4,63, the phenyl ring protons as a pair of doublets (A_2B_2 pattern) at δ 6.95 and 7.91, and the C-7 and C-8 protons as an unresolved single peak at δ 7.86. The corresponding values for (III.125) in the same solvent were δ 4.86, δ 6.91, and 7.98, and δ 7.48 and 7.76. The separation of the C-7 and C-8 resonances in (III.125) but not (III.124) was of interest insofar as it suggested a long-range interaction between positions 7 and 10. The UV spectra of (III.124) [λ_{max} (pH 13) 283 nm (ε 23,000); λ_{max} (pH 1) 251 nm (ε 16,000), 305 (11,000)] and (III.125) [λ_{max} (pH 13) 305 nm (ε 26,000); λ_{max} (pH 1) 250 nm (ε 17,000), 307 (12,000)] revealed a significant bathochromic displacement of the 283 nm peak of (III.125) at pH 13 to 305 nm in (III.124). This presumably reflects the influence of 10-Me substitution on the 4-aminobenzoyl chromophore.



(III.140) R^1 = (5'-phosphono-2'-deoxyuridyl)methyl, R^2 = H
(III.141) R^1 = H, R^2 = Et

In the preparation of the multisubstrate analogue (III.140) [39], diethyl 8-deazafolate (III.126) was reduced catalytically with PtO_2 in ethanolic HCl to obtain a 60% yield of the tetrahydro derivative (III.141). Condensation of (III.141) with 2'-*O*-acetyl-5'-[bis(2,2,2-trichloroethyl)phosphono]-5-bromomethyl-2'-deoxyuridine in dry DMF in the presence of $NaHCO_3$, followed by phosphate deprotection with Zn/Cu couple and acetylacetone, and finally ester hydrolysis, afforded a 41% overall yield (3 steps) of (III.140).

Because of the need to prove beyond doubt that the alkylation of (III.126) by the bromomethyluridine derivative occurred at position 5, a model reaction sequence was carried out on the 10-nitroso analogue, (III.128). This compound was prepared from (III.126) in 80% by treatment with $NaNO_2$-HCl, and was then reduced catalytically and condensed with 2',5'-di-*O*-acetyl-5-bromomethyl-2'-deoxyuridine. The product of this reaction and the one formed from (III.126) via the opposite sequence (i.e., by alkylation followed by nitrosation) proved to be indistinguishable. The NMR spectrum of (III.128), taken in d_6-DMSO, showed the $CH_2N(NO)$ protons as a singlet at δ 5.41 (deshielding effect by N=O), the phenyl ring protons as a pair of

doublets at δ 7.88 and 8.01, and the C-7 and C-8 protons as a pair of doublets at δ 7.92 and 8.10. Ultraviolet spactra [λ_{max} (pH 13) 274 nm (ε 21,000), 334 sh (7,800); λ_{max} (pH 7) 272 nm (ε 22,500), 294 sh (13,000); λ_{max} (pH 1) 250 nm (ε 24,500), 285 (15,000)] were consistent with N^{10}-nitroso substitution.

Biological activity data for 8-deazafolic acid (III.124) and several of its reduced derivatives have been reported against bacteria [37a] and against human tumour cells in culture [37b]. Enzyme inhibition data for these compounds have also been reported [37a,37b]. In assays against *S. faecium* and *L. casei* in the presence of 2.2 nM folic acid (1 ng/ml), the IC_{50} of (III.124) was found to be 0.32 and 1.3 nM, respectively. The potency of (III.124) was equal to that of MTX against *S. faecium*, but 60-fold lower against *L. casei*. The dihydro derivative of (III.124), prepared by sodium dithionite reduction but not chemically characterized, was approximately twice as active as (III.124) against *S. faecium*, but half as active against *L. casei*. The tetrahydro derivative obtained by catalytic hydrogenation was as active as the dihydro compound against *L. casei*, but was 8-fold less active against *S. faecium*. Both (III.124) and its reduced derivatives were more active than MTX against highly MTX-resistant strains of the organisms, though resistance could not be said to have been fully overcome. None of the compounds could support growth. As expected, these compounds were very weak inhibitors of bacterial dihydrofolate reductase ($IC_{50} > 10\ \mu M$), and were similarly ineffective as inhibitors of thymidylate synthase. In assays against human epithelial carcinoma cells (Hep.2) in culture [37b], (III.124) was found to have an IC_{50} of 23 μM as compared with 0.001 μM for MTX. Interestingly, the 5,10-methenyl derivative (III.139) showed somewhat greater activity, with an IC_{50} of 8 μM. Reversal experiments were performed in order to determine whether growth inhibition by (III.139) was due to an antipurine effect. Normal growth was observed in the presence of 11 μM folinic acid or a combination of 147 μM hypoxanthine and 41 μM thymidine, but not in the presence of 41 μM thymidine alone. Hypoxanthine by itself afforded only partial protection. These results were consistant with purine synthesis inhibition as the basis of action of (III.139), since this chemically labile 5,10-methenyl derivative might be converted intracellularly to the 10-formyl-5,6,7,8-tetrahydro derivative (III.138), a likely competitive antagonist of 10-formyltetrahydrofolate in purine synthesis. On the other hand, it was difficult to explain the fact that (III.138) itself, with an IC_{50} value of 28 μM, was 3.5-fold less active against Hep.2 cells than (III.139). Further work is needed to resolve this inconsistency.

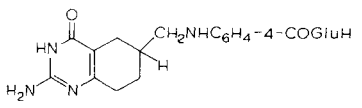
Inhibition assays against human TS showed (III.140) to be potent competitive inhibitor [39]. With dUMP as the variable substrate, the K_i(app) was

58 nM (K_i(app)/K_m = 0.02), whereas with 5,10-methylenetetrahydrofolate as the variable substrate the K_i(app) was 250 nM (K_i(app)/K_m = 0.005). The non-phosphorylated (nucleoside) as well as 5′-*O*-methylphosphate (nucleotide) analogues of (III.140) were also prepared and tested as TS inhibitors, but were less effective by several orders of magnitude. Because of its large size and multiplicity of ionized substituents, (III.140) is unlikely to be taken up efficiently into cells and is therefore viewed primarily as an enzyme probe, rather than as a potential therapeutic agent.

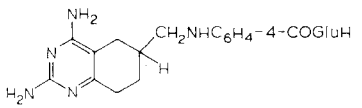
5,8-Dideaza compounds

2,4-Diamino derivatives

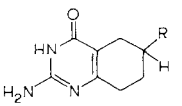
The earliest reported work on quinazoline (5,8-dideazapteridine) analogues of folic acid and the classical 2,4-diaminopteridine antifolates appears to have been that of the Baker group on 5,8-dideaza-5,6,7,8-tetrahydrofolic acid (III.141A) [40a,] and 5,8-dideaza-5,6,7,8-tetrahydroaminopterin (III.141B) [40b]. For the synthesis of (III.141A), 2,4-dicarbomethoxycyclohexanone was elaborated in several steps to 2-amino-5,6,7,8-tetrahydro-4(3*H*)-oxoquinazoline-6-carboxylic acid (III.141C) and thence to the alcohol (III.141D). The latter was converted to (III.141A) by successive reaction with thionyl chloride and condensation with *N*-(4-aminobenzoyl)-L-glutamic acid. The overall yield in this eight-step sequence was modest, and the product could not be very rigorously purified or characterized. For the synthesis of (III.141B), 4-oxocyclohexanecarboxaldehyde dimethyl acetal was elaborated in several steps, either with the cyano enol ether (III.141E) or by a more direct fusion reaction with cyanoguanidine, to the 2,4-diacetamido-5,6,7,8-tetrahydroquinazoline derivatives (III.141F) and (III.141G). Condensation of (III.141G) with *N*-(4-aminobenzoyl)-L-glutamic acid in acetic acid in the presence of hydrogen and platinum oxide, followed by hydrolysis of the crude coupling product with 0.4 M NaOH (30 min, steam bath), gave a 28% yield (two steps) of the desired deprotected 2,4-diamino compound (III.141B).



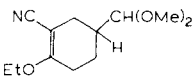
(III.141A)



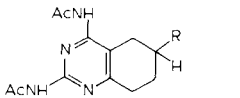
(III.141B)



(III.141C) R = COOH
(III.141D) R = CH_2OH



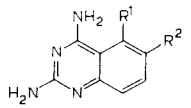
(III.141E)



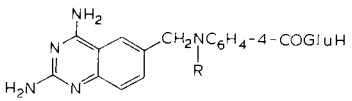
(III.141F) R = $CH(OMe)_2$
(III.141G) R = CHO

In antibacterial assays [40b], the 2-amino-4(3*H*)-oxo compound (III.141A) was found to be 30-fold less active than aminopterin as an inhibitor of the growth of *S. faecium*. In contrast, the 2,4-diamino compound (III.141B) was 6-times more potent than aminopterin. Qualitatively similar differences were observed against *P. cerevisiae*. Unfortunately, attempts to further exploit the strong antifolate activity of (III.141B) for cancer chemotherapy were thwarted when the compound was found to be extremely toxic *in vivo* (Mead, J.A.R., personal communication). Interestingly, however, the possibility of using leucovorin as a rescue agent to protect animals from the toxic effects of (III.141B) appears not to have been explored.

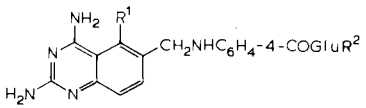
The earliest successful preparation of aromatic quinazoline analogues of the classical 2,4-diaminopteridine antifolates was that of Davoll and Johnson [40c], involving reductive condensation of diethyl *N*-(4-aminobenzoyl)-L-glutamate with either 2,4-diaminoquinazoline-6-carboxaldehyde (III.142) or 2,4-diaminoquinazoline-6-carbonitrile (III.143) in the presence of Raney Ni to give the diester (III.146). Hydrolysis of (III.146) gave 5,8-dideazaAMT (III.147). The overall yield (III.147) starting from (III.142) was 60%, whereas from (III.143) it was only 9%. However, since the aldehyde had to be prepared from the nitrile in two extra steps, the nitrile route was actually the shorter one. The same approach was also used to obtain the 5-Me analogue (III.148) and the 5-Cl analogue (III.149) via diesters (III.150) and (III.151).



(III.142) R^1 = H, R^2 = CHO
(III.143) R^1 = H, R^2 = CN
(III.144) R^1 = Me, R^2 = CN
(III.145) R^1 = Cl, R^2 = CN



(III.154) R = Me
(III.155) R = CHO



(III.146) R^1 = H, R^2 = Et
(III.147) R^1 = R^2 = H
(III.148) R^1 = Me, R^2 = H
(III.149) R^1 = Cl, R^2 = H
(III.150) R^1 = Me, R^2 = Et
(III.151) R^1 = Cl, R^2 = Et
(III.152) R^1 = H, R^2 = *t*-Bu
(III.153) R^1 = Cl, R^2 = *t*-Bu

The pioneering work of Davoll and Johnson [40c] was extended by Hynes *et al.* [41] and then by Jones [42]. The Hynes group [43, 44] also improved the synthesis of (III.153) and (III.155) by using the di-*t*-butyl rather than diethyl ester of *N*-(4-aminobenzoyl)-L-glutamic acid in the reductive coupling step. The coupling products, diesters (III.158) and (III.159), were converted to (III.154) and (III.155), respectively, by treatment with trifluoroacetic acid at room temperature for 1 h. The combined yield for the two steps was 57% for (III.153) and 38% for (III.155). Use of the acid labile t-butyl ester group afforded a cleaner product and avoided possible racemization of the amino acid side chain during alkaline hydrolysis. 5,8-DideazaAMT (III.147) was converted to 5,8-diazaMTX (III.154) in 87% yield by reaction with formaldehyde and $NaCNBH_3$, and to the N^{10}-formyl derivative (III.155) in 70% yield by reaction with formic acid and acetic anhydride.

The ability of 5,8-dideaza analogues of AMT and MTX to inhibit DHFR has been reported by several groups. Sources of enzyme in these studies have included *S. faecium*, *L.*casei, normal rat liver, murine L1210 leukaemia cells, and murine neuroblastoma cells. In assays with DHFR from rat liver and *S. faecium* [43], the 5-Me analogue (III.148) was found to be a 4-fold better inhibitor than the 5-unsubstituted compound (III.147). Activity against the mammalian and bacterial enzyme was similar for each compound. Calvert *et al.* [45] also examined (III.147) and (III.148), as well as the 5-Cl analogue (III.149), for the ability to inhibit rat liver DHFR, but did not find 5-substitution to have much effect on potency. Inhibition of DHFR by 5,8-dideazaMTX (III.154) was measured with L1210 enzyme and was compared to the effect of MTX and (III.155). The IC_{50} of both N^{10}-methyl compounds was approximately 0.05 μM, while that of the N^{10}-formyl compound was somewhat higher.

An interesting feature of the 5,8-dideaza compounds was uncovered [45] in assays against folate pathway enzymes other than DHFR. While the greatest potency of these compounds was clearly against DHFR and was relatively negligible against methionine synthetase and serine hydroxymethyltransferase, 5-substitution appeared to have a favourable effect on TS inhibition. The IC_{50} value of 5,8-dideazaAMT (III.147) against TS from Yoshida rat sarcoma cells had a rather high value of 100 μM, but that of (III.148) was 4.3 μM and that of (III.149) was only 0.25 μM. Thus, introduction of a 5-Cl substituent gave a 400-fold increase in affinity for this enzyme, suggesting that other 5-substituted analogues might be of interest as dual inhibitors of DHFR and TS.

A DHFR variant ('form 2') with greatly diminished affinity for MTX has been isolated in pure state from a MTX-resistant L5178Y murine leukaemia

cell line, and has been characterized with respect to its affinity for 2,4-diaminoquinazoline analogues of MTX and AMT [46, 47]. The IC_{50} values of both 5,8-dideazaAMT (III.147) and 5,8-dideazaMTX (III.154) were found to be 10- to 20-fold lower than the IC_{50} of MTX against this altered enzyme. The IC_{50} values of the 5-Me analogue (III.148) and 5-Cl analogue (III.149), however, were 70- to 80-fold lower, indicating that lipophilic 5-substituents have a favourable effect on binding to the active site of the form 2 enzyme. 5-Formylation at position 10, on the other hand, did not have this favourable effect, as (III.155) and MTX had almost the same IC_{50} value. The results of this study provided encouraging evidence that other 2,4-diamino antifolates structurally modified to enhance lipophilicity might find use in the treatment of MTX resistance when the latter is due to the emergence of a tumour cell population whose DHFR has an altered amino-acid sequence at the active site.

Early recognition of the potent cytotoxic properties of 5,8-dideazaAMT (III.147) came from the work of Hutchison *et al.* [48], who reported one line of L1210 cells to be 4.5-fold more sensitive to (III.147) than to MTX, while another line with somewhat different glutamine/asparagine requirements was 12-fold more sensitive to the quinazoline. An unusual feature of these experiments was that cell growth was determined after 7 days of drug treatment, rather than after 48 to 72 h as is more customary. The IC_{50} value of (III.147) under these conditions of prolonged exposure was 0.002 μM, whereas Hynes and Garrett [41] later found the IC_{50} value for 48 h of treatment to be 0.02 μM. It appears from the published data that the effect of (III.147) is more time-dependent than that of MTX, so that this compound is more potent than MTX after long exposure but less potent after shorter treatment.

In assays against cultured L1210/R81 cells [43], a resistant mutant with a severe defect in MTX active transport combined with a 35-fold increase in DHFR with normal MTX affinity [49], (III.147) was found to have an IC_{50} value of 46 μM as compared with 205 μM for MTX. Similar incomplete cross resistance was observed between MTX and the 5-substituted quinazolines (III.148) and (III.149). From these results it would seem that the 5,8-dideaza compounds may be taken up better than MTX by the MTX-resistant cells.

The effect of substitution at position 10 in the 5,8-dideaza series has been examined in L1210 and L1210/R81 cells and also in four human colon carcinoma cell lines [43, 44]. The IC_{50} of (III.154) was lower than that of (III.147) against both L1210 and L1210/R81 cells, and the same trend was observed in the human colon carcinoma lines. Compound (III.154) was more potent than MTX in these assays, but the 10-formyl analogue (III.155) was less potent. It thus appears that the rank order for cytotoxicity among the 5,8-

dideaza compounds with a glutamate side-chain is 10-Me > 10-H > 10-CHO. With respect to 5-substitution, Calvert *et al.* [45] have found little or no difference among (III.147), (III.148) and (III.149) as inhibitors of L1210 cell growth. More recently, Susten *et al.* [50] reported (III.149) to be almost 10-fold more potent than (III.147) or (III.148) against L1210 cells. The reasons for these divergent findings are unclear. In the recent studies of Hynes *et al.* [43, 44] against four gastrointestinal adenocarcinoma lines in culture, the 5-Cl analogue (III.149) (IC_{50} = 1.9–3.2 nM) was found to be only slightly more potent than either the 5-Me analogue (III.148) (IC_{50} = 3.4–5.0 nM) or the 5-H analogue (III.147) (IC_{50} = 3.5–4.2 nM). The effect of a 5-substituent on *in vitro* growth inhibition may thus vary depending on the tumour.

Comparison of the uptake of (III.147) and MTX in cultured L1210 cells [45] confirmed that the quinazoline was able to accumulate to a higher level than MTX when the cells were exposed to the same concentration of drug (4 μM) for 40 min. Competitive uptake experiments suggested that (III.147) shares a common active transport mechanism with MTX and folinic acid. As expected, high concentrations of folinic acid (50 μM) fully protected the cells from the effects of (III.147). At lower folinic acid concentrations, however, there was only partial protection; thus, 5 μM folinic acid (a 100-fold molar excess) was fully protected the cells from 0.05 μM (III.147), whereas 0.5 μM folinic acid (only a 10-fold excess) did not. Cells were also completely protected from 0.05 μM (III.147) with 100 μM thymidine and 100 μM hypoxanthine, and greater protection was achieved with 5 μM folinic and 100 μM thymidine than with 5 μM folinic acid alone. It was concluded from these results that (III.147) does not kill cells solely via DHFR inhibition, but rather via concomitant inhibition of DHFR and TS.

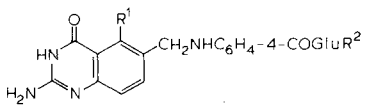
In vivo antitumour data consistent with the *in vitro* results have been reported for the 5,8-dideaza analogues of AMT and MTX, at least where the relative dose potency of the 5-unsubstituted *vis à vis* the parent drug is concerned [48]. However, it should be noted that while the optimal doses of (III.147) and (III.154) in mice with L1210 leukaemia were found to be lower than the optimal dose of MTX, the therapeutic benefit of the quinazolines as measured by the increase in lifespan (ILS) was either comparable to, or lower than, that of MTX. The optimal doses of (III.147) and MTX on a q2d × 5 schedule were found to be 0.2 and 1.5 mg/kg, respectively, with both compounds giving a 63% ILS. In another study [41] in which the optimal doses (III.154) and MTX on a q3d × 5 instead of qd × 5 schedule were found to be 2.75 and 10 mg/kg, respectively, the ILS values for the two drugs again proved to be of the same order. The 5-chloro analogue (III.149) gave a 50% ILS at 0.09 mg/kg (q2d × 5), its optimal dose, while the 5-methyl analogue (III.148) gave only

a 38% ILS at the same dose [51]. Thus, while the 5,8-dideaza compounds for the most part showed *in vitro* potency, this was not true *in vivo*, suggesting that 5-substitution in these analogues produces adverse host effects. Although the reason for the low efficacy of (III.148) and (III.149) has not been elucidated, it is tempting to speculate that 5-substitution in the quinazolines may be detrimental to polyglutamylation (as appears to be true in the 8-deaza series), or that the increased lipophilicity of the 5-substituted compounds may have an unfavourable effect on distribution to tumour versus host tissue.

Evidence that the quinazolines (III.147)–(III.149) (and presumably their diethyl esters) all have the same mode of action as MTX has been obtained *in vivo* by Hutchinson *et al.* [51] with a subline of L1210 leukaemia resistant to MTX and a second subline resistant to 6-mercaptopurine (6MP). Complete cross-resistance was observed between MTX and the quinazolines (III.147)–(III.149) against the L1210/MTX line, whereas all four compounds showed a marked increase in activity against the L1210/6MP line. For example, while a 63% ILS was obtained at 0.2 mg/kg (q3d × 5) with (III.147) against L1210 leukaemia, when the same compound was tested in mice with the L1210/6MP tumour a dose of 0.1 mg/kg (q3d × 7) produced a 133% ILS. This was similar to MTX, which gave a 150% ILS against the parental L1210 line but a 263% ILS against the L1210/6MP mutant. Similar results were found with the 5-substituted analogues (III.148) and (III.149). Since 6MP resistance is associated with HGPRTase deficiency, it would appear that inability to salvage purines ought to potentiate the antitumour action of all these compounds.

*2-Amino-4(3*H*)-oxo derivatives*

5,8-Dideazafolic acid (III.156) was first described in the literature by Davoll and Johnson [40a], who isolated it unintentionally during an attempt to form 5,8-dideazaAMT (III.147) by alkaline hydrolysis of the coupling product of diethyl *N*-(4-aminobenzoyl)-L-glutamate and 2,4-dibenzamido-6-bromomethylquinazoline. The desired cleavage of the amide and ester groups was accompanied by hydrolysis of the 4-amino group. Realizing that the 4-amino group in 2,4-diaminoquinazoline was base-labile, the authors then prepared 2-amino-4(3*H*)-quinazolinone-6-carbonitrile from the corresponding 2,4-

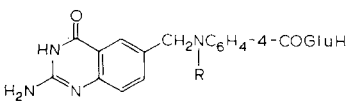


(III.156) $R^1 = R^2 = H$ (III.159) $R^1 = Cl, R^2 = Et$
(III.157) $R^1 = H, R^2 = Et$ (III.160) $R^1 = Me, R^2 = H$
(III.158) $R^1 = Me, R^2 = Et$ (III.161) $R^1 = Cl, R^2 = H$

diamine and allowed it to react with diethyl *N*-(*p*-aminobenzoyl)-L-glutamate in the presence of hydrogen and Raney Ni to obtain diethyl 5,8-dideazafolate (III.157). Alkaline hydrolysis then yielded (III.156). Similarly prepared were the diethyl esters (III.148) (31%) and (III.149) (10%), which yielded 5-methyl-5,8-dideazafolate (III.160, 89%) and 5-chloro-5,8-dideazafolate (III.161) (93%), respectively, on treatment with aqueous ethanolic NaOH at room temperature.

Also in 1970, Bird *et al.* [52] reported in a brief but significant paper that (III.156) and the N^{10}-methyl analogue (III.162) were potent inhibitors of *S. faecium* (or *S. faecalis* as this organism was then called), with IC_{50} values of 1.3 and 0.16 nM, respectively, in the presence of 0.91 nM folic acid in the medium. The IC_{50} of MTX in the same assay was 0.44 nM. This was a milestone discovery, since it established that potent antifolate activity could be achieved even with a compound of the 2-amino-4(3*H*)-oxo type when the nitrogens in ring B were deleted. Leucovorin (0.84 nM) alone afforded only slight protection from the toxic effects of these compounds, whereas a combination of leucovorin (0.84 nM) and thymidine (41 nM) was strongly protective. Thus, cell-killing was associated with blocked thymidylate biosynthesis. Enzyme inhibition assays showed (III.156) and (III.162) to be 500- to 1000-fold less potent than MTX as inhibitors of *S. faecium* DHFR and 10- to 15-fold less potent as inhibitors of pigeon liver DHFR. In sharp contrast, in assays against bacterial (*E. coli*) TS, (III.156) had an IC_{50} of 0.75 μM and was 93-fold more potent than MTX (IC_{50} = 70 μM), while (III.162) had an IC_{50} value of 0.098 μM and was 76-fold more potent than (III.156) and 714-fold more potent than MTX. Since (III.162) was a better inhibitor of TS than of DHFR, it was concluded that thymidylate synthesis was being inhibited directly at the level of TS, rather than as a secondary byproduct of tetrahydrofolate depletion. Moreover, alkyl substitution at N^{10} was clearly an important determinant of anti-TS activity.

The original synthesis of 5,8-dideazafolates by Davoll and Johnson [40c] suffered from an important flaw, in that the intermediate 2-amino-4(3*H*)-quinazolinone-6-carbonitriles were prepared from the corresponding 2,4-diamines and could therefore be contaminated with trace quantities of 5,8-dideazaAMT or its 5-substituted congeners. Since the latter were known to



(III.162) R = Me
(III.163) R = CHO

be exceedingly potent inhibitors of DHFR, biological data obtained with the 2-amino-4(3*H*)-oxo compounds had to be suspect. With this in mind, Acharya and Hynes [53] devised an efficient alternative synthesis of (III.156) which avoided this problem. Condensation of ethyl 4-methylanthranilate with guanidine afforded the 2-amino-6-methyl-4(3*H*)-quinazolinone (80%), which was successively blocked on the 2-amino group by pivaloylation (75%), subjected to free radical bromination at the 6-methyl group with 1,3-dibromo-5,5-dimethylhydantoin (84%), deprotected with methanolic HCl (95%), condensed with diethyl *N*-(4-aminobenzoyl)-L-glutamate in the presence of triethylamine (52%), and hydrolyzed in 0.1 M NaOH (67%) to obtain (III.156) with an overall yield of 15–20%. The same series of reactions using diethyl *N*-[4-(*N*-methylamino)benzoyl]-L-glutamate yielded the 5,8-dideaza-N^{10}-methylfolic acid (III.162). 5,8-Dideaza-N^{10}-formylfolic acid (III.163) was subsequently obtained from (III.156) in 75% yield by heating with 98% formic acid [52].

As part of the major effort undertaken at the Chester Beatty Research Institute in the area of quinazoline antifolates, Jones [42] synthesized 5,8-dideazafolate (III.156), 5-methyl-5,8-dideazafolate (III.160), and 5-chloro-5,8-dideazafolate (III.161) by a synthesis which basically followed the Davoll scheme, except for one important modification: 2,4-diamino-quinazoline-6-carbonitriles were converted into 2-amino-4(3*H*)-oxoquinazoline-6-carbonitriles in boiling 2 M HCl rather than in alkali. Hydrolysis of the 4-amino group under these conditions was more than 99% complete, thus ensuring the purity of the final products.

Data on the inhibitory effects of (III.156), (III.160), and (III.161) (prepared by the Davoll method) against partially purified DHFR from rat liver and *S. faecium* were reported in 1975 by Hynes *et al.* [54]. Against rat liver enzyme, the IC_{50} values of these quinazolines were 0.023, 0.0037 and 0.012 μM, respectively (versus < 0.001 μM for MTX); against the *S. faecium* enzyme, the corresponding IC_{50} values were 1.6, 0.43, and 0.30 μM (versus 0.0014 μM for MTX). Thus, deletion of the 5- and 8-nitrogens from folic acid seemed to give compounds with increased affinity for the mammalian enzyme. Moreover, N^{10}-formylation was surprisingly well tolerated. However, the 2-amino-4(3*H*)-oxo compounds were substantially less potent than the corresponding 2,4-diamines in the DHFR assay. For example, as discussed in the preceding section, the IC_{50} values of 5,8-dideazaAMT against DHFR from pigeon liver and *S. faecium* were 0.0017 and 0.0062 μM, respectively. Thus, most, if not all, the observed anti-DHFR activity of (III.156) in this initial study could have been the result of minor contamination by 5,8-dideazaAMT (as little as 5% in the assay with pigeon liver enzyme). Because of this

problem, considerable care has been taken since then to show (for example, by HPLC) that any 5,8-dideazafolate tested for biological activity is over 99% pure.

Dedhar *et al.* [47] have evaluated the activity of (III.156), (III.162), and (III.161) as inhibitors of 'type 2 DHFR' (low MTX affinity) from MTX-resistant murine L5178Y lymphoma cells. The IC_{50} values of the three 2-amino-4(3*H*)-oxoquinazolines were > 1.0, 1.7, and 0.2 μM (versus 0.026, 0.032, and 0.24 μM for the corresponding 2,4-diaminoquinazolines and 0.45 μM for MTX in the same assay). Thus, the N^{10}-unsubstituted and N^{10}-methyl substituted 2-amino-4(3*H*)-oxoquinazolines were some 50-fold less potent than their 2.4-diamino counterparts. In contrast, the 10-CHO analogues in the 2-amino-4(3*H*)-oxo and 2,4-diamino series were equipotent, and were more potent in each case than the 10-H or 10-Me compounds. The significance of these subtle structure–activity correlations with regard to binding to type 2 DHFR are not yet clear.

In a recent study [55], (III.156), (III.160), and the 10-CHO derivative (III.163) were tested against DHFR from HCT-8 human colon tumour cells, and were found to have IC_{50} values of 0.05, 0.35, and 0.009 μM, respectively, as compared with 0.0013 μM for MTX and 0.00065 μM for 5-methyl-5,8-dideazaAMT (III.148). An interesting feature of these results was the rather high potency of (III.163), which was consistent with the data obtained against DHFR from L5178Y murine lymphoma cells by Dedhar *et al.* [47] and was reminiscent of the anti-DHFR activity reported a number of years ago for N^{10}-formylfolate [56].

Inhibition of purified TS from murine L1210 cells by (III.156), (III.162) and (III.163) has been studied [57]. The IC_{50} values followed the order 10-Me ((III.162), 0.13 μM) < 10-H ((III.156), 3.4 μM) < 10-CHO ((III.163), 9.8 μM), as compared with 0.6, 5.0 and > 10 μM for the corresponding 2,4-diaminoquinazolines. The 2-amino-4(*H*)-oxo compounds were thus more potent TS inhibitors in every case than the 2,4-diamines.

The results of an experiment comparing the activity of (III.156) and the chemically synthesized γ-monoglutamyl derivative (III.156) + G_1 as inhibitors of TS from HCT-8 human colon tumour cells were recently reported by McGuire *et al.* [55]. Whereas the IC_{50} of (III.156) was 8 μM, that of (III.156) + G_1 was only 0.3 μM. This 27-fold increase in binding affinity was consistent with the known enhancement of TS substrate activity for 5,10-methylenetetrahydrofolate on addition of a second glutamyl residue. Inhibition of TS from HCT-8 cells was also found to be decreased 2-fold relative to (III.156) on introduction of a 5-Me group, and 15-fold on N^{10}-formylation. Compounds (III.156) and (III.162) were both good substrates for partially

purified folylpolyglutamate synthetase from HCT-8 cells. The N^{10}-formyl derivative (III.163), while it was likewise a substrate, appeared to be less efficiently polyglutamylated than the N^{10}-unsubstituted compounds. It was concluded that the ability of dideazafolate analogues to inhibit cellular TS activity was probably dependent on their ease of polyglutamylation, in addition to other factors such as rate of uptake.

Advantage has been taken of the binding affinity of (III.162) and (III.163) for TS to prepare affinity columns for the purification of this enzyme. One laboratory [58] utilized a column of the N^{10}-formyl derivative (III.163) immobilized on aminoethyl-Sepharose to purify TS to electrophoretic homogeneity from L1210 leukaemia cells. A second group [59] prepared a similar column from the N^{10}-methyl derivative (III.162) and used it to purify TS from neonatal mouse liver.

The activity of (III.156), (III.162) and (III.163) as inhibitors of the growth of a panel of human gastrointestinal carcinoma cell lines (HuTu80, HT29, SW480, WIDR) in culture has been reported [60]. The ranges of IC_{50} values against the four cell lines for each of the quinazolines were as follows: (III.156), 0.6–1.1 μM; (III.162), 1.2–1.9 μM; (III.163), 38–84 μM. Thus, in contrast to the trend in DHFR inhibition assays, the ability of the 10-H and 10-Me compounds to kill cells was greater than that of the 10-CHO analogue. Growth inhibition data have also been reported for (III.156), (III.162) and (III.163) against HCT-8 human colon tumour cells; the IC_{50} values were 2.0, 1.3 and 13 μM, respectively [55]. Thus, there was little change in activity against these cells on introduction of a 5-Me substituent but a considerable attenuation of activity on N^{10}-formylation.

Calvert *et al.* [45] conducted comprehensive studies of the *in vitro* and *in vivo* action of compounds (III.156), (III.162) and (III.163). In assays against TS from Yoshida sarcoma, the IC_{50} values of the quinazolines followed the order 5-H ((III.156), 0.29 μM; $K_i = 67$ nM, competitive with respect to 5,10-methylenetetrahydrofolate) > 5-Cl ((III.163), 5.5 μM) > 5-Me ((III.162), 22 μM). The same order of potency was followed against rat liver DHFR (III.162), $IC_{50} = 0.25$ μM; $K_i = 0.35$ nM; (III.163), $IC_{50} = 0.63$ μM; (III.162), $IC_{50} = 1.1$ μM), but activity was greater against DHFR than against TS. In comparisons of 2,4-diamino versus 2-amino-4(3*H*)-oxo substitution, activity against DHFR was consistently greater among the 2,4-diamines, but activity against TS was dependent on substitution at position 5. Thus, with (III.147) and (III.156) (both 5-H), anti-TS activity was 345-fold greater with the 2-amino-4(3*H*)-oxo compound, while with (III.148) and (III.162) (both 5-Me), anti-TS activity was 88-fold greater with the 2,4-diamine. With (III.149) and (III.163) (both 5-Cl), on the other hand, anti-TS activity was approximately

the same. These curious structure-activity correlations will undoubtedly become clearer as more knowledge is gained concerning the three-dimensional structure of TS and, more specifically, the interaction of various quinazoline inhibitors with amino acid residues at the enzyme active site.

Inhibition studies with quinazolines (III.156), (III.162) and (III.163) were also carried out by the Chester Beatty group [45] against three other enzymes of the folate pathway, methionine synthetase, tetrahydrofolate formylase, and serine hydroxymethyl transferase. Less than 50% inhibition could be achieved against any of these enzymes at drug concentrations as high as 0.1 mM. Thus, the action of these compounds seemed to be confined to the two folate-requiring enzymes most sensitive to other antifolates, namely TS and DHFR. In order to determine more precisely which of these enzymes was, in fact, the most likely target, reversal studies using cultured L1210 cells were performed. The concentrations required to inhibit cell growth by 50% in a 48 h incubation were found to be 3.4 μM for (III.156), 2.7 μM for (III.162), and 4.2 μM for (III.163) (as compared with *ca.* 0.02 μM for the more potent 2,4-diamines). The toxicity of 10 μM (III.156) to L1210 cells was completely prevented in the presence of 10 μM folinic acid or 50 μM thymidine, indicating that the cell-killing effect of the 2-amino-4(3*H*)-quinazolinone is due to TS inhibition. In contrast, the toxicity of 0.05 μM 5,8-dideazaAMT (III.147) was only partially prevented with 5 mM folinic acid, 100 μM thymidine, or a combination of the two, but was completely overcome when 100 μM hypoxanthine was added. Thus, while the former compound was acting chiefly on TS, the latter behaved in a manner consistent with a dual locus of action, i.e., DHFR and TS.

In order to gain information about the likely mechanism of membrane transport of the 2-amino-4(3*H*)-oxoquinazoline antifolates, Calvert *et al.* [45] examined the effect of varying concentrations of (III.156) (up to 100 μM) on the accumulation of 4 μM [^{3}H]MTX, 4 μM [^{3}H]folate, and 4 μM [^{3}H]-methyltetrahydrofolate over 40 min by L1210 cells. Curiously, there was no inhibition of the uptake of any of these compounds by (III.156). The authors concluded that (III.156) might not utilize either the folate or reduced folate transport pathway, but did not indicate what alternative transport route for this compound might exist. Transport of (III.156) via the folate carrier would seem a likely route of entry into cells, but might be inefficient in comparison with transport of the natural substrate. This would explain the inability to block the uptake of 4 μM folate even with 100 μM (III.156).

In vivo experiments in normal and tumour-bearing mice were also performed with (III.157) [45]. In contrast to 5,8-dideazaAMT (III.147), which had a single-dose LD_{50} in mice of 2–4 mg/kg and produced significant antitumour activity against L1210 leukaemia in mice (see above), (III.157) was non-toxic

when given i.p. at doses of up to 480 mg/kg and was devoid of antitumour activity at this dose.

The possibility that the N^{10}-formyl derivative (III.163) might serve as a substrate for two important enzymes of the *de novo* purine pathway that utilize reduced folates as their natural substrates, namely 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase and glycinamide ribonucleotide (GAR) transformylase, was examined [61]. While the affinity of (III.163) (K_m(app) = 29 μM) for AICAR transformylase appeared to be greater than that of the natural substrate 10-formyltetrahydrofolate (K_m(app) = 68 μM), the reaction was slow, resulting in a 750-fold lower V_{rel}/K_m(app) ratio for the quinazoline. The affinity of (III.163) (K_m(app) = 1.9 μM) for GAR transformylase was likewise several times greater than that of the natural substrate, in this case 5,10-methenyltetrahydrofolate (K_m(app) = 8.9 μM). However, (III.163) was also used rather efficiently in the reaction by GAR transformylase, resulting in a 4-fold *higher* V_{rel}/K_m(app) for the quinazoline than for 5,10-methenyltetrahydrofolate. The authors concluded from these results that the 5,10-methenyl structure is not needed for GAR transformylase activity.

In a recent paper [62], inhibition of aminoimidazole-4-carboxamide ribonucleotide transformylase (AICAR TFase) by the 5,8-dideazafolic acid (III.156) and its 5-methyl (III.160), 10-methyl (III.162) and 10-formyl (III.163) analogues was reported. The apparent K_i values for the 5-unsubstituted compounds were all in the 10–30 μM range, as compared with a substrate K_m of 68 μM for the 'natural' diastereomer of 10-formyltetrahydrofolate. In contrast, the apparent K_i for the 5-methyl analogue (III.160) was > 200 μM, indicating that 5-substitution was unfavourable for AICAR TFase binding in this series. While the relatively high K_i values of these compounds probably precluded a significant biochemical role at cytotoxic concentrations, it was speculated that the corresponding polyglutamates might in fact be more potent. This was supported by data for the γ-glutamyl derivatives of (III.156) and (III.162), whose K_i values were found to be 3.5 and 1.8 μM, respectively. Data for longer oligoglutamates would obviously be of interest.

CHANGES IN REGION C

Modifications in the so-called 'bridge region' of the classical antifolate structure have been of two general types. In the first type, the 2,4-diaminopteridine moiety was left intact and changes were made only in the bridge; in the second,

modifications were made in the pyrazine ring (Region B) as well. As will be seen below, this has been, and is likely to remain, fertile ground for research.

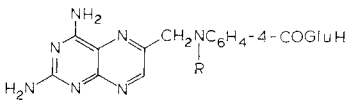
COMPOUNDS MODIFIED ONLY IN THE BRIDGE

Substitution on N^{10}

2,4-Diamino derivatives

Replacement of the N^{10}-methyl group of MTX by larger, more lipophilic substituents has been studied for its effects on DHFR binding, membrane transport, *in vitro* cell growth inhibition, and *in vivo* antitumour activity [14, 63–65].

Routes previously developed for the synthesis of MTX and AMT were generally followed to obtain these compounds [14, 63]. Ethyl 4-(tosylamino)benzoate was allowed to react with alkyl (Et, *n*-Pr, *n*-Oct) or aralkyl (CH_2Ph, CH_2CH_2Ph) halides in the presence of NaH, and the products were treated stepwise with base, thionyl chloride, diethyl L-glutamate, and 30% HBr-AcOH-PhOH to form *N*-[4-(*N*-alkylamino)benzoyl]-L-glutamic acid derivatives. Condensation with 2,4-diamino-6-bromomethylpteridine in DMA at 50–55 °C, followed by ester hydrolysis with 1 M NaOH in MeOH, then gave the N^{10}-substituted analogues (IV.1)–(IV.5). In the case of the N^{10}-propargyl analogue (IV.6), use of a *p*-toluenesulphonamide intermediate was precluded by the incompatibility of the propargylamine group with HBr-AcOH. Therefore, diethyl L-glutamate was treated with propargyl bromide, the monoalkylated product (after removal of unchanged starting material and dialkylated product) was condensed with 1,4-diamino-6-bromomethylpteridine, and the ester groups were hydrolyzed. In an alternative route to the N^{10}-benzyl derivative (IV.4) [64], diethyl *N*-(4-aminobenzoyl)-L-glutamate was reductively alkylated with benzaldehyde in the presence of sodium cyanoborohydride, the ester groups in the resulting *N*-benzyl derivative were hydrolyzed, and the product was coupled to 2,4-diamino-6-bromomethylpteridine. The NMR spectrum of (IV.4) in d_6-DMSO showed the aromatic protons of the *p*-aminobenzoyl group as a pair of A_2B_2 doublets at δ 6.83 and 7.29, and the C-7 proton as a singlet at δ 8.63. These chemical shifts had the



(IV.1) R = Et (IV.4) R = CH_2Ph
(IV.2) R = *n*-Pr (IV.5) R = CH_2CH_2Ph
(IV.3) R = *n*-Oct (IV.6) R = $CH_2C{\equiv}CH$

expected values for AMT analogues, and suggested no unusual interaction involving the π-orbitals of the N^{10}-benzyl moiety.

The effect of N^{10}-substitution on DHFR binding was measured with enzyme from L1210 cells. The N^{10}-ethyl and N^{10}-*n*-propyl analogues (IV.1) (K_i = 0.0039 nM) and (IV.2) (K_i = 0.0034 nM) were comparable to MTX (K_i = 0.0043 nM) [14, 60], whereas (IV.3), with its longer, more lipophilic N^{10}-*n*-octyl substituent, showed 350-fold decreased affinity (K_i = 1.4 nM) [65]. Remarkably, the N^{10}-benzyl and N^{10}-(2-phenylethyl) analogues (IV.4) (K_i = 3.5 μM) and (IV.5) (K_i = 1.9 μM) were almost 10^6-fold less potent than MTX, suggesting that the L1210 enzyme, at least when assayed under cell-free conditions, is intolerant of bulky hydrophobic substitution at N^{10}. In contrast to their low activity against the L1210 enzyme, however, (IV.4) and (IV.5) were approximately equipotent with MTX against a bacterial DHFR (from *Diplococcus pneumoniae*). The reason for the poor binding of (IV.4) and (IV.5) to the isolated L1210 enzyme is unclear. In assays using purified DHFR from *L. casei*, (IV.2), (IV.3), and (IV.6) had IC_{50} values of 12–10 nM, and were equipotent with MTX [63]. The IC_{50} values of these compounds against purified TS from the same organism were in excess of 20 μM.

Bacterial growth inhibition data have been reported for the *n*-octyl and propargyl analogues (IV.3) and (IV.6) in comparison with MTX [63]. The IC_{50} values (IV.3) against *S. faecium* and *L. casei* were 0.72 and 0.030 nM. The corresponding values for (IV.6) were 0.41 and 0.027 nM, respectively, while those for MTX were 0.2 and 0.03 nM. Thus, the antibacterial activities of (IV.3) and (IV.6) were similar to MTX and were consistent with their anti-DHFR activity. Neither compound overcame MTX resistance in *L. casei*/MTX. However, *S. faecium*/MTX was about 50-fold less resistant to (IV.3) than to MTX.

The ability of compounds (IV.1)–(IV.6) to inhibit the growth of several mammalian cell lines has been evaluated in culture [63, 65]. In one study using L1210 cells [65], the ethyl and *n*-propyl analogues (IV.1) and (IV.2) had IC_{50} values of 1.7 and 1.8 nM, while the benzyl and 2-phenylethyl analogues (IV.4) and (IV.5), with IC_{50} values of 34 and 30 nM, were 10- to 20-fold less potent. The IC_{50} values of AMT and MTX in the same experiment were 0.8 and 5.4 nM, respectively. Thus, (IV.1) and (IV.2) were less potent than AMT but more potent than MTX. In the second study [63], the *n*-propyl, *n*-octyl, and propargyl analogues (IV.2), (IV.3) and (IV.6) were found to have IC_{50} values of 2.2, 217 and 1.4 nM, respectively, while AMT and MTX had IC_{50} values of 1.2 and 2.7 nM. Thus, while activity was retained in the three-carbon propargyl analogue, a 100-fold decrease in toxicity occurred with the *n*-octyl analogue, despite its high affinity for isolated DHFR. This was in surprising

contrast to the benzyl and 2-phenylethyl analogues, which were more toxic than the *n*-octyl analogue, even though they were much poorer inhibitors of the enzyme.

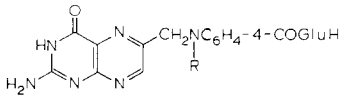
Comparisons of the membrane transport properties of the *n*-propyl, *n*-octyl, and propargyl analogues (IV.2), (IV.3), and (IV.6) were made in L1210 leukaemia, S180 sarcoma, Ehrlich carcinoma, and normal intestinal epithelial cells [63]. In L1210 cells, (IV.2) and (IV.6) appeared to have an affinity for the transport carrier similar to that of MTX, while the affinity of (IV.3) was somewhat decreased. In S180 and Ehrlich cells, on the other hand, all three compounds seemed to be transported 5- to 10-fold better than MTX. Like MTX, compounds (IV.2), (IV.3), and (IV.6) were all transported less well in normal intestinal epithelial cells than in tumour cells. The K_i(S180)/K_i(epithelium) ratio was 0.008 for (IV.2), 0.001 for (IV.3), and 0.006 for (IV.6), as compared with 0.03 for MTX. In contrast, the K_i(L1210)/K_i(epithelium) ratio for (IV.3) was 0.004. The 30-fold difference in the K_i(S180)/K_i(epithelium) ratio between MTX and (IV.3) augured well for the therapeutic index of this compound against the S180 tumour *in vivo*, whereas the smaller difference in the K_i(L1210)/K_i(epithelium) ratio suggested that (IV.6) and MTX ought to have similar *in vivo* activity, provided that plasma clearances are likewise similar (*vide infra*).

In vivo antitumour tests in mice were carried out with compounds (IV.2), (IV.3) and (IV.6) against L1210 leukaemia and with compound (IV.6) against Sarcoma 180 [63]. Treatment of L1210 leukaemic mice with 288 mg/kg (q2d × 5) of either (IV.2) or (IV.6) produced survival increases of 133 and 143%, respectively, while MTX on the same schedule gave a survival increase of 152% at 15 mg/kg. The *n*-octyl analogue (IV.3) at 144 mg/kg (the highest dose reported) was inactive, probably because of low solubility. Against the S180 ascites tumour, the propargyl analogue (IV.6) at 288 mg/kg (q2d × 5) gave only a 67% ILS, versus 82% for MTX at its optimal dose (15 mg/kg). However, when the schedule was changed to qd × 5, treatment with 36 mg/kg of (IV.6) produced a 129% ILS, whereas treatment with the optimal dose of MTX (6 mg/kg) gave only a 78% ILS. Thus, the therapeutic efficacy of (IV.6) was more schedule dependent than that of MTX. Data for the *n*-propyl and *n*-octyl analogues in this system were not reported. The schedule dependence of (IV.6) appeared to be related to its more rapid plasma clearance in comparison with MTX.

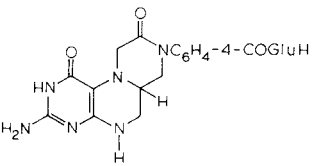
*2-Amino-4(3*H*)-oxo derivatives*

The effect of N^{10}-substitution in folic acid and tetrahydrofolic acid has similarly been of interest because of the possibility that this would give rise

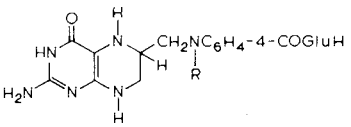
to compounds with antifolate activity. Temple *et al.* [66] reported in 1982 that treatment of folic acid with several simple acyl chlorides in *N,N*-dimethylacetamide at room temperature occurred selectively at N^{10}. Compounds obtained in this manner, in yields ranging from 30 to 80%, were the N^{10}-acetyl, N^{10}-chloroacetyl, N^{10}-(3-chloropropionyl), and N^{10}-ethoxalyl derivatives (IV.7)–(IV.10). Catalytic hydrogenation of (IV.8) in trifluoroacetic acid at atmospheric pressure led to a mixture of N^5,N^{10}-(2-oxoethylene)tetrahydrofolate (IV.11), N^{10}-acetyltetrahydrofolate (IV.12), and N^5,N^{10}-methenyltetrahydrofolate (IV.15). The last compound was presumed to form by carbon–carbon bond cleavage of 5,10-chloroethenyltetrahydrofolate (IV.16). Similar reduction of (IV.7) and (IV.9) afforded only N^{10}-acetyltetrahydrofolate (IV.12) and N^{10}-(3-chloropropionyl)tetrahydrofolate (IV.13), respectively, with no evidence of ring closure to N^5,N^{10}-ethenyltetrahydrofolate (IV.17) from (IV.7) or of N^5,N^{10}-(3-oxoethylene)tetrahydrofolate (IV.18) from (IV.9). Attempted hydrogenation of N^{10}-ethoxalylfolic acid (IV.10) gave a 7:3 mixture of the N^{10}-substituted tetrahydro derivative (IV.14) and the corresponding N^5-substituted isomer, but the compounds were not individually separated and characterized.



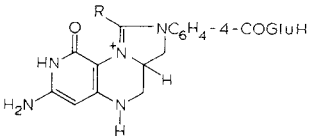
(IV.7) R = CH_3CO
(IV.8) R = $ClCH_2CO$
(IV.9) R = $ClCH_2CH_2CO$
(IV.10) R = EtO_2CCO



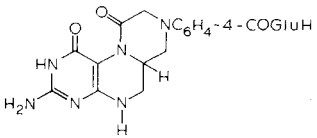
(IV.11)



(IV.12) R = CH_3CO
(IV.13) R = $ClCH_2CH_2CO$
(IV.14) R = EtO_2CCO



(IV.15) R = H
(IV.16) R = $ClCH_2$
(IV.17) R = CH_3

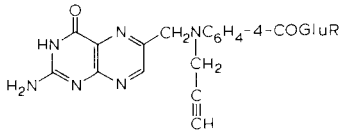


(IV.18)

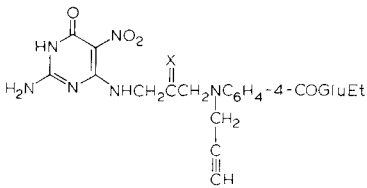
The 10-chloroacetyl derivative (IV.8) was tested as an inhibitor of TS from L1210 cells and DHFR from beef liver [66]. There was no significant effect on activity at the highest concentration of inhibitor tested (100 μM), and only weak inhibition of DHFR (IC_{50} = 57 μM). Slight inhibition of Hep.2 cell growth in culture was observed (IC_{50} = 8 μM), but no activity against L1210 leukaemia in mice. It was clear from these results that simple N^{10}-substitution on folic acid was not likely to produce useful antitumour activity.

Following the discovery of the tight binding of N^{10}-propargyl-5,8-dideazafolate (CB3717, IV.287; *vide infra*) to thymidylate synthase (TS), it became of obvious interest to examine the effect of N^{10}-propargyl substitution in folic acid. Moreover, it was of interest to determine how N^{10}-propargyl substitution would affect the biological activity of 7,8-dihydrofolic and especially 5,6,7,8-tetrahydrofolic acid. It was proposed that if N^{10}-propargylfolate (or the 7,8-dihydro derivative) turned out to be substrates for DHFR, and if the resulting 5,6,7,8-tetrahydro compound with the natural configuration at C-6 proved to be a good inhibitor of TS, selective toxicity to MTX-resistant tumours with high levels of DHFR might be possible [66]. This scenario, it should be noted, was identical to the one proposed more than 20 years ago as the basis for the synthesis of homofolic acid (IV.26); see next section). N^{10}-Propargylfolic acid (IV.19) was found to be most conveniently prepared via a Boon-Leigh synthesis involving as key intermediates the nitro oxime (IV.21) and nitro ketone (IV.22). Treatment of (IV.22) first with sodium hydrosulphide in DMF-H_2O and then with alkali resulted in spontaneous ring closure and ester hydrolysis to form N^{10}-propargyl-7,8-dihydrofolic acid (IV.23), which on treatment with 5% $KMnO_4$ afforded (IV.19) (*ca.* 30% yield based on (IV.22)). The nitro oxime (IV.21) was prepared from *N*-(3-bromo-2-oxopropyl)phthalimide by reaction with diethyl *N*-[4[(*N*-propargyl)amino]-benzoyl-L-glutamate, followed by removal of the phthalimido group with anhydrous hydrazine. A second route to (IV.19) involved analogous synthesis of N^{10}-propargylpteroic acid (IV.24), by mixed anhydride coupling to diethyl L-glutamate to form the diethyl ester (IV.20), and alkaline hydrolysis. It was also possible to prepare (IV.20) from 2-amino-6-bromomethylpteridin-4(3*H*)-one and diethyl *N*-[4-(*N*-propargylamino)benzoyl]-L-glutamate in hot DMSO or DMA, but the yield in this coupling reaction was very low, probably because of poor solubility. The structure of (IV.19) was supported by its UV spectrum [λ_{max} (0.1 M NaOH) 255 nm (ε 27,200), 291 (26,100), 366 (9,500)], and by its NMR spectrum in trifluoroacetic acid solution, which showed the propargyl CH_2 group as a characteristic singlet at δ 4.2. Reduction of (IV.19) with sodium dithionite afforded the 7,8-dihydro derivative (IV.23) (λ_{max} 285 nm), which on further reduction with DHFR from *L. casei* afforded the

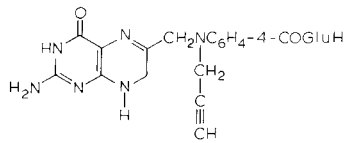
5,6,7,8-tetrahydro derivative (IV.25) (λ_{max} 300 nm), with C-6 assumed to have the natural configuration.



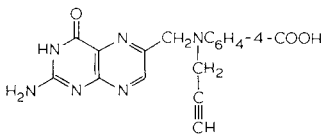
(IV.19) R = H
(IV.20) R = Et



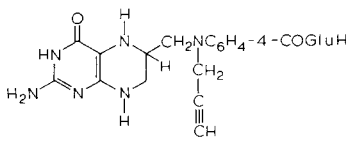
(IV.21) X = NOH
(IV.22) X = O



(IV.23)



(IV.24)



(IV.25)

In assays against purified TS from *L. casei*, IV.19, IV.23, and IV.25 were found to have IC_{50} values of 3.9, 50, and 32 μM, respectively, while the quinazoline analogue CB3717 had an IC_{50} value of 0.013 μM [67]. It appeared that replacement of the quinazoline moiety in CB3717 by a pteridine was very detrimental to TS binding. Moreover, reduction of ring B to either the dihydro or tetrahydro form led to an even greater loss of affinity. Thus, even though (IV.23) was a DHFR substrate as had been hoped, the resulting tetrahydro derivative (IV.25) was a poorer inhibitor of TS than the unreduced compound and had less than 0.1% of the activity of the nonreduced compound. Because it was possible that polyglutamylation might enhance binding of (IV.19) to TS, the ability of this compound to inhibit TS activity in intact as well as permeabilized L1210 cells was examined. Activity was very low in the intact cells (25% inhibition at 3 μM), and was approximately the same in the permeabilized cells (IC_{50} = 7.5 μM) as it was against the isolated enzyme. These results could mean (i) that (IV.19) was not a good substrate for the FPGS in the cells, or (ii) that it did form polyglutamates, but that the affinity of these poly-

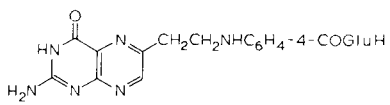
glutamates for TS was no greater than that of the monoglutamate. Further work would have to be done to distinguish between these possibilities.

In addition to the enzyme inhibition assays described above (IV.19) was tested as a growth inhibitor against MTX-sensitive and MTX-resistant *L. casei* and *S. faecium* [67]. Interestingly, while IV.19 had $< 0.1\%$ of the potency of CB3717 and $< 0.01\%$ of the potency of MTX against *L. casei*, there was much less difference among the three agents against *S. faecium*, and in fact the tetrahydro derivative (IV.25) appeared to be somewhat more potent against the latter organism than either CB3717 or MTX. The reason for the high level of activity of (IV.25) against *S. faecium* remains unclear at present, but may be due to inhibition of some folate-utilizing enzyme other than TS, such as GAR transformylase.

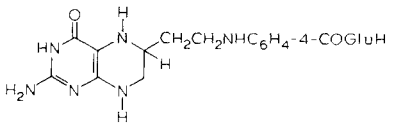
Insertion of extra carbons between C^9 and N^{10}

Chemical synthesis

In 1964, Goodman *et al.* [68] reported the synthesis of homofolic acid (HFA, (IV.26)), the first example of a folic acid analogue with a bridge lengthened by insertion of a carbon atom. The original rationale for the synthesis of HFA was that, especially in tumours resistant to MTX because of increased DHFR activity, this compound would be reduced to 5,6,7,8-tetrahydrohomofolic acid (H_4HFA, (IV.27)). Because of its structural similarity to 5,6,7,8-tetrahydrofolic acid, H_4HFA was regarded as being potentially a competitive inhibitor of reduced folate metabolism. Selective reduction of HFA to the growth inhibitory metabolite H_4HFA by MTX-resistant tumour cells would represent a form of self-immolation. Of the various enzymes in the metabolism of reduced folates, the one considered to be the most likely target for inhibition was TS, which meant that the end result of treatment with HFA would be 'thymineless death'. As will be discussed below, this hypothesis proved incorrect and H_4HFA was shown to be, in fact, an inhibitor of purine synthesis. However, the original concept behind the synthesis of HFA retains its basic validity and continues to be admired as an example of rational drug design based on biochemical principles.

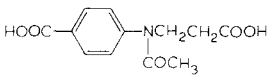


(IV.26) (HFA)

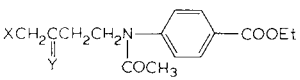


(IV.27) (H_4HFA)

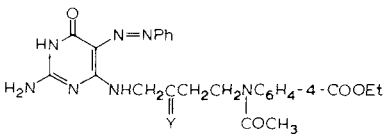
Chemistry. The original synthesis of HFA by DeGraw and co-workers [69] was an adaptation of the classic Boon-Leigh pteridine synthesis [11], wherein a key step was the reductive cyclization of a 2-amino-5-phenylazo-6(1*H*)-oxopyrimidine with a suitable carbonyl-containing moiety at position 4 to a 7,8-dihydropteridine. Through a lengthy series of standard chemical reactions, ethyl *p*-aminobenzoate was converted to the acid (IV.28), the α-substituted ketones (IV.29)–(IV.31), and finally the semicarbazone (IV.32). A similar scheme using a formyl instead of acetyl group to protect the aniline nitrogen was abandoned because it gave less satisfactory results. Condensation of (IV.32) with 2-amino-4-chloro-5-phenylazo-6(1*H*)-oxopyrimidine in DMF containing 2,4,6-trimethylpyridine as the acid scavenger (or with a 2:1 excess of (IV.32) without 2,4,6-trimethylpyridine) afforded (IV.34). On successive removal of the semicarbazone group with acid and reduction of the phenylazo group by catalytic hydrogenation (IV.34) was converted to the dihydropteridine (IV.35). The reductive cyclization step was at times erratic, but was reported to give yields as high as 83%. Oxidation of (IV.35) to ethyl N^{11}-acetylhomopteroate (IV.36) was accomplished in 93% yield with H_2O_2. Vigorous alkaline treatment of (IV.36) (0.25 M NaOH, 1 h on the steam bath) hydrolyzed both the ester and amide blocking groups, giving homopteroic acid



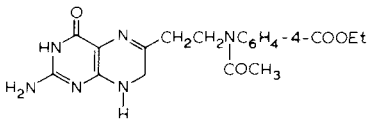
(IV.28)



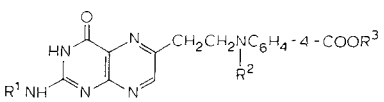
(IV.29) X = CH_2Cl, Y = O
(IV.30) X = CH_2N_3, Y = O
(IV.31) X = CH_2NH_2, Y = O
(IV.32) X = CH_2NH_2, Y = $NNHCONH_2$
(IV.33) X = CH_2OAc, Y = O



(IV.34) Y = $NNHCONH_2$



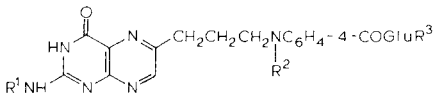
(IV.35)



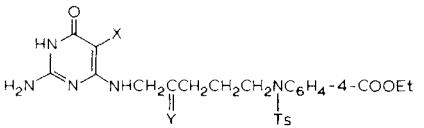
(IV.36) R^1 = CH_3CO, R^2 = CH_3CO, R^3 = Et
(IV.37) R^1 = R^3 = H, R^2 = CH_3CO
(IV.38) R^1 = CH_3CO, R^2 = CF_3CO, R^3 = H
(IV.39) R^1 = R^2 = H, R^3 = Et
(IV.40) R^1 = R^2 = R^3 = H

(IV.40). Reprotection of the nitrogens in (IV.40) was accomplished best by a two-step sequence consisting of heating first with trifluoroacetic anhydride and then with acetic anhydride. This led to the N^2-acetyl-N^{11}- trifluoroacetyl derivative (IV.38), which could be converted to HFA by mixed anhydride coupling to diethyl L-glutamate and subsequent ester and amide hydrolysis with strong base (0.1 M NaOH, 25 min on the steam bath). Like folic acid, HFA was light-sensitive. The overall yield for the final coupling and deprotection steps was 15–20%. As expected, the UV spectrum [λ_{max} (pH 13) 255 nm (ε 24,600), 281 (19,450), 365 (7,880)] was similar to that of folic acid.

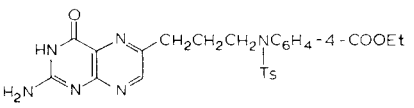
Bishomofolic acid (IV.41) was prepared by a scheme similar to the one leading to HFA, except that a different route had to be used to obtain the homologated α-aminosemicarbazone (IV.43) [70]. Ethyl 4-(*N*-tosyl)aminobenzoate was alkylated with 1-bromo-4-pentene, the product was epoxidized, the epoxide ring was cleaved selectively with sodium azide by attack at the terminal carbon, the resulting secondary alcohol was oxidized to a ketone, and the azido group was reduced catalytically to form an α-amino ketone, which was further elaborated to either (IV.43) or the 5-nitro analogue (IV.44). Reductive ring closure of (IV.43) followed directly by oxidation with H_2O_2 afforded ethyl N^{12}-tosylbishomopteroate ((IV.45), 46% yield after recrystallization). Alkaline ester cleavage (75% yield), protection of the 2-amino group by acetylation (83%), and mixed anhydride coupling to diethyl L-glutamate (46%) gave the protected bishomofolic acid diethyl ester (IV.42). Upon ester and amide cleavage (1.0 M KOH in MeOH, 30 min reflux) followed directly by tosyl group cleavage (30% HBr in acetic acid and phenol), (IV.42) was converted to (IV.41) in 63–87% yield. The UV spectrum of purified material [λ_{max} (pH 13) 253 nm (ε 25,600), 280 (19,900), 365 (7,200)] was very similar to the spectrum of HFA. The generality of this scheme was demonstrated by



(IV.41) $R^1 = R^2 = R^3 = H$
(IV.42) $R^1 = CH_3CO$, $R^2 = Ts$, $R^3 = Et$



(IV.43) $X = NNC_6H_5$, $Y = NNHCONH_2$
(IV.44) $X = NO_2$, $Y = NNHCONH_2$



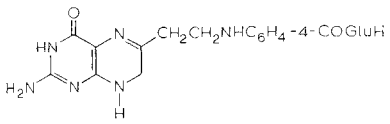
(IV.45)

substituting 1-bromo-3-butene for 1-bromo-4-pentene, which led to HFA. Use of a tosyl group in place of acetyl to protect the anilino nitrogen was also judged to be a considerable advantage.

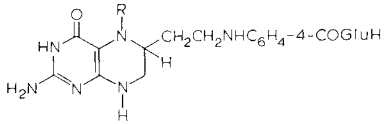
In an improved synthesis of HFA (IV.26) described in 1972 [71], 2,4,5-triaminopyrimidin-6(1*H*)-one was allowed to react at 60 °C under alkaline conditions (pH 9.0–9.4) for 14 h with 1-acetoxy-4-[*N*-acetyl-*N*-(*p*-carbethoxyphenyl)amino]-2-butanone (IV.33) and the product was treated with H_2O_2 in HCl to obtain crude ethyl N^{11}-homopterate (IV.39) in 90% yield. Alkaline hydrolysis of (IV.39) gave homopteroic acid (IV.40, 87%), and attachment of the side-chain proceeded in 70% overall yield to give HFA. The intermediate ketone (IV.33) was synthesized by a series of routine steps from methyl vinyl ketone and ethyl p-aminobenzoate. A noteworthy feature of the synthesis was the regiospecificity of the coupling reaction between the diaminopyrimidine and α-acetoxyketone systems, which gave exclusively a 6-substituted pteridine. The mechanism was proposed to involve initial formation of an α-acetoxy Schiff's base, followed by deacetylation, and Lobry de Bruyn-Van Eckenstein transformation to an α-aminoaldehyde which immediately undergoes ring closure [71]. The high pH at which the reaction is conducted appears to be critical, not only because it ensures that the ketone reacts first with the most basic 5-amino group of the pyrimidine, but also because it may block unwanted purine formation via nucleophilic attack of the 6-amino group at the initially formed C=N bond.

Chemical reduction of the pyrazine ring in HFA was accomplished by procedures well known from folic acid chemistry: reduction with sodium dithionite yielded 7,8-dihydrohomofolic acid (H_2HFA) (IV.46), while catalytic hydrogenation led to the 5,6,7,8-tetrahydro derivative (IV.27) [68, 72]. Similarly, reaction of H_4HFA (IV.27) with formaldehyde in the presence of sodium borohydride afforded the 5-methyl derivative (IV.47) (5-MeH_4HFA) [73], while reaction with formic acid and DCC yielded the 5-formyl derivative (IV.48) (5-FmH_4HFA) [74]. A procedure using dimethylamine borane in formic acid at low temperature has also been devised as a means of converting HFA directly to (IV.48). A 60% yield was isolated by this method, along with other lesser products which probably included (IV.47) [75]. Contrary to initial findings, this procedure works less well with folic acid than with HFA, and is therefore not useful in its present form as a preparative route to leucovorin [76]. Reaction of HFA with a hot mixture of formic acid and acetic anhydride has been found to yield, by analogy with folic acid, the 10-formyl derivative (IV.49) [77], while formylation of H_4HFA leads to 5,11-methenyl-5,6,7,8-tetrahydrohomofolate (IV.49A) [77, 78]. The latter product is also obtained from (IV.49A) in aqueous HCl, though formation of the six-membered ring is

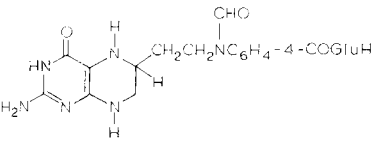
less facile than closure of the five-membered imidazolidine ring from leucovorin. The 5-methyl derivative (IV.47) was originally prepared with the idea that it might be more stable than H_4HFA, and might therefore be easier to purify and formulate for preclinical and clinical use. On chemical grounds one might question whether addition of the 5-methyl group can substantially alter stability, since N^5 in (IV.47) is still an amine. It has been proposed that (IV.48), in which N^5 is an *amide*, ought to be superior to (IV.47) in this regard [74].



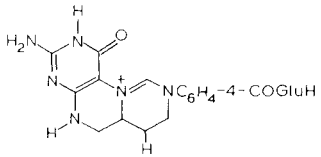
(IV.46) (H_2HFA)



(IV.47) R = Me (5-MeH_4HFA)
(IV.48) R = CHO (5-FmH_4HFA)



(IV.49)



(IV.49A)

Biological activity. The earliest evidence in conflict with the 'classical' model of the biochemical action of HFA (IV.26) and its reduced derivatives was that of Nichol and Hakala [79], who studied a murine Sarcoma 180 mutant cell line (AH/3000) which was 2,100-fold resistant to MTX in culture in comparison with the parent line (AH/S), and whose DHFR activity was increased approximately 200-fold. The IC_{50} value of HFA against the AH/3000 cells and the parental line (AH/S) was found to be 5.1 and 7 μM, respectively. Thus, in contrast to what the model predicted, the AH/3000 mutant did not show increased HFA sensitivity paralleling its increased DHFR activity. Shortly thereafter, the results of a study comparing the effects of MTX and HFA on [^{14}C]deoxyuridine incorporation into the DNA of cultured MTX-sensitive and MTX-resistant L1210 murine leukaemia cells were reported [80]. As expected, 1 h treatment with 10 μM MTX inhibited DNA labelling by 65% in L1210 cells but only 18% in the L1210/FR8 mutant, which was known to have elevated DHFR activity [81]. In the presence of 10 μM HFA, there was little effect on DNA labelling in either cell line, and the authors concluded that the biochemical site of action of reduced homofolates was probably not at the level of TS as originally postulated. Another study along the same lines was

that of Mishra and Mead [82] in 1972, in which [^{14}C]deoxyuridine, [^{14}C]orotate, and [^{14}C]formate incorporation into DNA was measured in spleens of mice with L1210 and L1210/FR8 leukaemia after treatment with H_4HF (533 mg/kg IP at 0, 8, and 22 h post–inoculation of tumour). Incorporation of [^{14}C]deoxyuridine and [^{14}C]formate into leukaemic spleen was decreased to 35–45% of controls in both groups, even though there was a 30-fold difference in DHFR activity between the L1210 and L1210/FR8 tumour. On the other hand, [^{14}C]orotate incorporation, while unaffected in mice with wild-type tumour, was decreased by 90% in animals with the DHFR overproducing variant. This led the authors to suggest that the site of action of reduced homofolates, at least in this tumour model, may lie somewhere between orotic acid and deoxyuridine.

The question of whether reduced homofolates could replace folates in one-carbon metabolism was a critical one with regard to the mode of action of these compounds. Kisliuk and Gaumont [83] observed that 7,8-H_2HFA (IV.46) had only about 2% of the substrate activity of dihydrofolate for *S. faecium* DHFR, while chemically derived (6-*R,S*)-L-H_4HFA (i.e., a mixture of the 'natural' and 'unnatural' stereomers at position 6) showed 25% of the substrate activity of (6-*R,S*)-L-tetrahydrofolate for serine hydroxymethyltransferase. The ensuing product, presumably the 5,11-methylene derivative of (6-*R,S*)-L-H_4HFA, had 12% of the substrate activity of 5,10-methylene-(6-*R,S*)-L-tetrahydrofolate for TS. It thus appeared that endogenous metabolism of HFA had the potential to convert it to products that could act, albeit not very efficiently, as false substrates for enzymes of the folate pathway. The important finding was also made, however, that enzymatically reduced homofolates, which presumably had the 'natural' configuration at position 6, did not behave the same way as those with the 'unnatural' configuration. That is, only the 'unnatural' reduced homofolates inhibited the growth of *S. faecium* while the diastereomers with the 'natural' configuration actually supported growth. Although these data were for bacterial rather than mammalian cells, they suggested that comparative *in vivo* experiments with HFA and chemically synthesized H_4HFA (or derivatives thereof) should be interpreted with caution. Since studies in two mouse tumour cell lines have shown that HFA cannot be used as a growth factor [84, 85], it appears that any H_4HFA formed *in situ* by DHFR is unlikely to be an efficient cofactor in the one-carbon metabolism of these mammalian cells.

An important clue to the biochemical locus of action of homofolates was provided in 1974 by Taylor and Hanna [86]. When TS activity was measured in intact *E. coli*, 80% inhibition could be achieved with 10 μM H_4HFA; in HeLa cells, however, there was only 50% inhibition of TS activity even at a

concentration of 150 μM, which was more than sufficient to completely suppress cell growth. Thus, while TS inhibition might contribute in a major way to the antibacterial action of HFA, it appeared that TS was not the primary target at concentrations toxic to mammalian cells.

More definitive information on the mechanism of action of HFA in mammalian systems came soon thereafter from work of Divekar and Hakala [87], who showed by reversal experiments that inhibition of S180 murine sarcoma cells in culture by HFA was probably associated with decreased synthesis of *N*-formylglycinamide ribotide (FGAR). When the cells were treated with 10 μM HFA alone, rates of cell growth rate and FGAR synthesis both declined to 30–40% of untreated controls. When 10 μM HFA was added in the presence of 50 μM hypoxanthine, however, cell growth returned to normal values while FGAR synthesis remained inhibited. Treatment with 50 μM hypoxanthine and 0.1 μM leucovorin caused both cell growth and FGAR synthesis to return to control levels. When concentrations of HFA greater than 50 μM were used, the combination of 50 μM hypoxanthine and 0.1 μM leucovorin was only partially effective. Since the *N*-formyl group in FGAR is biochemically derived from leucovorin via 5,10-methenyltetrahydrofolate, it appeared that at least some of the biological effects of homofolates were due to inhibition of the enzyme GAR transformylase, which converts glycinamide ribotide to FGAR in one of the earliest steps of the *de novo* purine pathway.

In an interesting study of the biochemical action of H_4HFA in L1210 cells, Scanlon *et al.* [85] found the IC_{50} value of the compound to be 50 μM as compared with 125 μM for HF itself. When cells depleted of folates by being grown for 3 weeks in folate-free medium were transferred to growth medium containing 10 mg/l of HFA (in place of folic acid) they did not grow. Somewhat surprisingly, however, folate-depleted cells incubated in the presence of HFA (10 mg/l), thymidine (10 μM), and hypoxanthine (50 μM) in folate-free medium still failed to grow. While this latter observation was difficult to reconcile with either TS inhibition or purine synthesis inhibition as a mechanism of action, it was evident that, in L1210 cells at least, HFA could not replace folic acid as a growth factor. In an effort to clarify this issue at the biochemical level, H_4HFA was tested for substrate and/or inhibitor activity against several folate pathway enzymes partially purified from L1210 cells. Interestingly, H_4HFA was utilized almost as efficiently as tetrahydrofolate at equimolar concentration in reactions catalyzed by thymidylate synthase, 5,10-methylenetetrahydrofolate reductase, 5,10-methylenetetrahydrofolate dehydrogenase, and 10-formyltetrahydrofolate synthetase. In the assays against TS from L1210 cells, H_4HFA was found to have a substrate K_m(app) of 66 μM as compared with 45 μM for tetrahydrofolate. As expected from this

similarity in K_m(app) values, H_4HFA inhibited the utilization of tetrahydrofolate by TS. Depending on the method of analysis, the K_i was calculated to be 42 μM (slope method) or 26 μM (intercept method). Interestingly, however, the kinetics of inhibition were of the hyperbolic noncompetitive type, suggesting that 5,11-methylenetetrahydrohomofolate was acting by partial inhibition of two separate binding sites on the enzyme. A novel finding of this study was also that H_4HFA was used very inefficiently as a cofactor in the conversion of tetrahydrofolate and L-serine to 5,10-methylenetetrahydrofolate by serine hydroxymethyltransferase, and was in fact an inhibitor of the enzyme with an IC_{50} of 62.5 μM, suggesting that this was perhaps another site of biochemical action at the cellular level. Another interesting observation by these authors was that HFA treatment caused profound methionine depletion in the L1210 cells, which are known to be autotrophic for this amino acid [88]. However, the extent to which inhibition of methionine active transport contributes to the cytotoxicity of HFA to L1210 cells remains unclear.

The *in vivo* antitumour activity of HFA was first reported in 1966 by Mead *et al.* [89], who showed that subcutaneous treatment with just two 150 mg/kg doses of HFA on days 3 and 7 after tumour implantation gave a 30–45% ILS in mice with either wild-type L1210 leukaemia or the DHFR overproducing L1210/FR8 variant. Interestingly, prior chemical reduction of HFA to H_2HFA and H_4HFA decreased activity against both tumours on the two-dose schedule, though the effect was more pronounced against the L1210 line. Daily drug administration resulted in increased survival. Subcutaneous 400 mg/kg doses of H_4HFA given daily until death to mice with L1210/FR8 leukaemia gave an 84% ILS, whereas similar treatment with 1.5 mg/kg of MTX (which led to a 157% ILS against L1210 leukaemia) had no effect. When leucovorin (20 mg/kg) was injected immediately before H_4HFA, antitumour activity disappeared completely. Attempts were made to use HFA and its reduced derivatives together with MTX, in the hope that combination regimens might be synergistic. Unfortunately synergism was not observed; indeed, the drugs appeared to be somewhat antagonistic. While it was found subsequently [90] that the timing of administration of the two drugs was important, the overall conclusion remained that DHFR inhibition profoundly diminished the effectiveness of homofolates, presumably because the enzyme is required to maintain the drug in the tetrahydro form inside the cell.

It was suggested that a critical determinant of the antitumour activity of HFA might be the nutritional status of the host animal [90]. When mice with MTX-resistant L1210/FR8 leukaemia who were on a regular diet received 200 mg/kg of H_4HFA, starting on day 7 and continuing daily until death, a 42% increase in survival was observed. With animals on a folate-deficient

diet, on the other hand, antitumour activity was completely lost. The effect was dose-dependent, and was not observed when a 100 mg/kg dose of H_4HFA was used. Moreover, a folate-deficient diet at this lower drug dose appeared to increase, rather than decrease, activity against the wild-type tumour. Thus, a clearcut conclusion from this nutritional experiment was difficult to draw.

Elucidation of the precise biochemical mode of action of HFA and its reduced derivatives has been hampered by a number of problems. Not the least of these has been a lack of rigorous studies comparing the metabolic fate of these compounds in different types of normal and malignant cell. The National Cancer Institute group [91] showed in mice that the extent of conversion of H_2HFA (IV.46) to H_4HFA (IV.27) in liver was dramatically decreased by pre-treatment with MTX. Nahas and Friedkin [92} compared the matabolic fate of H_2HFA and dihydrofolate in cultured MTX-sensitive L1210 cells and an MTX-resistant mutant (L1210/MTX) with 13-fold elevated DHFR activity. While dihydrofolate was efficiently reduced to tetrahydrofolate by both L1210 and L1210/MTX cells, recovery of intact drug from cells treated with (IV.27) was extremely low, even when the experiment was performed in ascites cells in mice. This was difficult to reconcile with the report [91] showing that (IV.46) is reduced extensively in liver. Additional experiments comparing the formation of (IV.27) from HFA or (IV.46) in tumour and non-tumour tissue of the same animal would help resolve these inconsistencies.

Some of the early biochemical and pharmacological work carried out with 5-MeH_4HF (IV.47) was based on the not necessarily valid assumption that this compound and H_4HF would act by the same mechanism. A pertinent finding by Taylor and Hanna [86] in this regard was that H_4HFA (IV.27) was an inhibitor of TS in *E. coli*, whereas (IV.47) was not. The latter was also a poorer inhibitor of TS in HeLa cells, giving only a 25% decrease in dTMP synthesis from dUMP at a concentration several times greater than the IC_{50} for cell growth inhibition. It was evident from this experiment that the primary site of growth-inhibitory action (IV.47), like that of (IV.27), was not at the level of TS. It remained to be determined, on the other hand, whether (IV.47) was merely a stable *congener* of (IV.27), or whether it was, at least in part, a *prodrug*. The latter could come about, for example, if (IV.47) replaced 5-methyltetrahydrofolate as a methionine synthetase substrate. Indeed, Taylor and Hanna [86] demonstrated this to be the case in *E. coli* and three representative examples of mammalian cells (HeLa, CHO, and rabbit kidney). Compound (IV.47) was found to be a methyl group donor, though its substrate activity was only about 10% of the substrate activity of 5-methyltetrahydrofolate. Although these results have to be viewed with caution because the

substrate and/or inhibitor properties of the individual 'natural' and 'unnatural' diastereomers of (IV.47) and 5-methyltetrahydrofolate were not compared, they nonetheless suggest that at least some of the biological effects of (IV.47) may be due to bioconversion to (IV.27).

As with all classical antifolates, transport and polyglutamylation are important aspects of the biochemical pharmacology of homofolates. Kinetic data on the transport of 5-MeH_4HF (IV.47) by various cells are sparse, though Goldman *et al.* [93] have reported that cultured Ehrlich cells appear to have two carrier-mediated transport pathways for this compound. At low concentrations, (IV.47) was taken up by a high-affinity carrier (low K_t) with a relatively low V_{max}. This carrier also transported 5-methyltetrahydrofolate, leucovorin, and MTX, and was sensitive to metabolic poisons. At concentrations of (IV.47) greater than 15 μM, uptake involved a second carrier which was insensitive to metabolic poisons, and had a lower affinity (high K_t) with a high V_{max}. Leucovorin was again a competitive inhibitor, but MTX was not. With regard to polyglutamylation, Moran *et al.* [94] have reported that HFA possesses only about half the substrate activity of folic acid or MTX toward mouse liver folylpolyglutamate synthetae. The number of glutamates added to HFA by the enzyme in intact tumour cells has not been determined. It is likely that this number is smaller than with MTX in view of the much higher doses of HFA required for a comparable *in vivo* antitumour effect. In a recent study, Sirotnak *et al.* [95] obtained kinetic data for the transport of (6*S*)-5-[^{14}C]MeH_4HFA (6*S*)-(IV.47)) and (6*R*)-5-[^{14}C]MeH_4HFA ((6*R*)-(IV.47)) in L1210 and L1210/R24 cells, the latter of which were MTX resistant by virtue of a transport defect. The K_m for influx in L1210 cells was approximately 3-times lower for (6*S*)-(IV.47) than for (6*R*)-(IV.47), while the V_{max} values were the same. Both compounds were much less efficiently taken up than MTX, and in fact resembled folic acid itself, rather than reduced folates, in their rate of uptake. Competitive transport experiments were also performed, and it was found that the K_i of (6*S*)-(IV.47) as an inhibitor of MTX was 2-fold lower than that of (6*R*)-(IV.47), while the K_i of (6-*R,S*)-(IV.47) had the expected intermediate value. Similar differences were observed when inhibition of folic acid transport was measured. As expected, the uptake of both diastereomers of (IV.47) was less efficient in L1210/R24 cells than in L1210 cells, though the differences were not dramatic. A more striking difference between (6*S*)-(IV.47) and (6*R*)-(IV.47), however, was that, whereas the initial uptake of (6*S*)-(IV.47) was rapid over the first 5 min and then exhibited a second, slower phase consistent with intracellular metabolism, the initial uptake of (6*R*)-(IV.47) was slower than that of (6*S*)-(IV.47) and a second phase did not occur. Efflux studies were also performed, and it was

observed that the rate of loss of (6*S*)-(IV.47) from L1210 cells pre-loaded with drug was half as rapid as that of (6*R*)-(IV.47). A reasonable interpretation of these contrasting results is that (6*S*)-(IV.47) is polyglutamylated and serves as a substrate for methionine synthetase, whereas (6*R*)-(IV.47) is metabolically inert.

Extensive studies to evaluate the antitumour spectrum of 5-MeH_4HFA (IV.47) were carried out in animals as part of a preclinical development program at the National Cancer Institute (Mead, J.A.R., personal communication). As with H_4HFA (IV.27), best results were obtained against MTX-sensitive and MTX-resistant L1210 murine leukaemia. In the most successful experiments, daily subcutaneous administration of (IV.47) from day 3 until death at a dose of 50 mg/kg gave a 183% ILS in mice with subcutaneously implanted L1210/MTX tumour. Statistically significant activity was also seen in mice with Lewis lung carcinoma (63% ILS at 400 mg/kg, qd × 9), P288 leukaemia (62% ILS at 400 mg/kg, qd × 9), colon 26 carcinoma (47% tumour growth inhibition at 800 mg/kg, q4d × 3), and colon 38 carcinoma (57% tumour growth inhibition at 200 mg/kg, q7d × 2). No activity was observed against P388 leukaemia or B16 melanoma.

As a test of the possibility that 5-MeH_4HFA (IV.47) and MTX might be synergistic, L1210 leukaemic mice have been subjected to concurrent treatment with (IV.47) (0–400 mg/kg, qd × 9) and MTX (0–60 mg/kg, q3d × 3) (Rosowsky, A., unpublished results). At every dose of MTX, concurrent treatment with (IV.47) interfered with normal weight gain and led to a smaller ILS than with (IV.47) or MTX alone. In another experiment, mice were treated daily for 1 week with 500 mg/kg of (IV.47) and then with a single 200–500 mg/kg dose of MTX. After 3 weeks, the animals treated with 500 mg/kg of MTX after (IV.47) pretreatment showed massive weight loss. In contrast, animals treated only with 500 mg/kg of MTX showed a smaller weight gain than untreated controls, but did not lose weight. These results suggested that the observed *in vivo* antagonism between (IV.47) and MTX might be due to greater depletion of stored folates from normal host tissues than from tumour by the (IV.47).

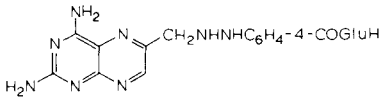
Pharmacokinetic and tissue distribution studies have been performed with H_4HFA (IV.27) in mice [89, 96–98] and monkeys [99, 100], and with 5-MeH_4HFA (IV.47) in mice, dogs, and monkeys [101, 102]. The plasma level of (IV.27) in monkeys 30 min after a single IV dose of 60 mg/kg was 250 μM [99]. By 1 h, this level had declined to 150 μM. Signs of decreased clearance began to appear after six daily doses of 60 mg/kg, suggesting impaired renal function. In mice, a 400 mg/kg IP bolus resulted in plasma levels of 1 mM at 0.5 h and 0.48 mM at 1 h. With (IV.47) [101, 102], a single 150 mg/m^2 i.v. dose

in dogs gave a plasma triphasic clearance curve with half-lives of 6 and 49 min for the α and β phase and 27 h for the terminal phase. Similar results were obtained at a higher dose of 1500 mg/m^2 except for the half-life of the terminal phase, which was increased to 45 h. In the monkey, the terminal phase had about the same half-life (13–15 h) regardless of whether the dose was 150 or 1500 mg/m^2. Binding to plasma proteins was 60–80% at both doses. The majority of the drug (50–90% in 24 h after a bolus dose of 150 mg/m^2) was excreted by the mouse, dog, and monkey in urine, and excreted drug appeared to be unchanged, suggesting that if any bioconversion to (IV.27) occurred, the metabolite was extensively retained. In all three species, drug levels at 1 h were highest in liver and kidney, and lowest in brain. Pharmacokinetic data have also been reported for (IV.47) in humans [103, 104]. In the first study, which used a low 15 mg/m^2 dose of radiolabelled drug given i.v. [103], triphasic plasma clearance was observed, with half-lives of 0.5, 6.4, and 87 h for the α, β, and γ phase, respectively. In the second study [104], in which patients on a Phase I protocol received i.v. doses ranging from 500 to 2600 mg/m^2 and real plasma levels of drug were measured by HPLC over 8 h, the data were fitted to a two-compartment model that gave half-lives of 0.8 h for the α phase and 5.6 h for the β phase. Plasma levels after 3–4 days of daily treatment did not show significant drug accumulation. Binding to plasma proteins was estimated to be 40–60% and was dose independent. By 24 h most of the administered drug (60–95%) could be accounted for in the urine. These results were consistent with the data reported in animals, and indicated that the drug is tolerated in 5-day courses of up to 2500 mg/m^2 per day.

As indicated in the preceding section, 5-FmH_4HFA (IV.48) has been proposed as a alternative reduced homofolate analogue, the properties of which might be qualitatively or quantitatively different from those of 5-MeH_4HFA (IV.47) [74]. In assays against L1210 cells in culture, the IC_{50} value of (IV.48) was found to be 5.1 μM, while those of HFA and (IV.47) were 16 and 5.6 μM, respectively. Thus, the growth inhibitory potencies of (IV.48) and (IV.47) were essentially the same. As with HFA and (IV.47), growth inhibition by (IV.48) was reversed by hypoxanthine alone but not by thymidine alone. This implicated *de novo* purine biosynthesis (most likely at the level of AICAR transformylase) as the site of action. In preliminary *in vivo* antitumour assays against MTX-resistant L1210/FR8 leukaemia in mice, (IV.47) and (IV.48) (both as chemically prepared mixtures of 'natural' and 'unnatural' diastereomers) gave a 53% ILS at 600 mg/kg (qd × 9). However, while the dose of (IV.47) could be increased to 1200 mg/kg, giving a 123% ILS, the same increase with (IV.48) was toxic (Mead, J.A.R., personal communication). Further studies, especially with the individual 'natural' and 'unnatural' diastereomers of (IV.48), would be of interest.

Insertion of C or N between N^{10} and the phenyl ring

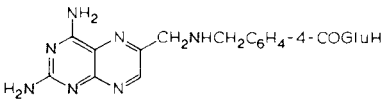
An early example of this class was 'azahomoaminopterin' (azahomoAMT, (IV.50)), which Slavik *et al.* [105] prepared in 1969 from 2,4-diaminopteridine-6-carboxaldehyde and *N*-(4-hydrazinobenzoyl)-L-glutamic acid by reductive coupling in the presence of sodium borohydride.



(IV.50)

In assays of mouse liver DHFR inhibition using dihydrofolate as the substrate, MTX gave an IC_{50} value of 0.029 μM, while (IV.50) gave an IC_{50} value of 0.74 μM. Thus, lengthening of the bridge by insertion of an extra N atom resulted in a 15-fold decrease in binding to DHFR. In assays using several other enzymes of the folate pathway, including serine hydroxymethyl transferase, (IV.50) was inactive even at 100 μM; however, marginal inhibition did occur at 400 μM.

A second example of bridge lengthening was contributed subsequently by the Slavik group [106] in the form of 'isohomoaminopterin' (isohomoAMT, (IV.51). In this instance, 2,4-diaminopteridine-6-carboxaldehyde was condensed with diethyl *N*-[4-(α-aminomethyl)benzoyl]-L-glutamate under catalytic hydrogenation conditions. The reduced adduct from this reaction was reoxidized with I_2-KI and the ester groups were removed with base to obtain a product formulated as (IV.51). It should be noted, however, that the reported C and N microanalytical data for this compound were not within acceptable limits, and that the published UV absorption data included unusual long wavelength peaks at 410 nm at pH 13 and at 400 nm at pH 1 which were inconsistent with a 2,4-diamino-6-alkylpteridine structure.



(IV.51)

Compound (IV.51) was tested as an inhibitor of mouse liver DHFR with either folate (at pH 5.5) or dihydrofolate (at pH 7.5) as the substrate. Competi-

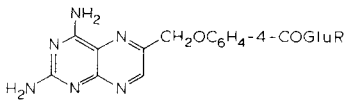
tive inhibition kinetics were obtained in both assays. With folate as the substrate the K_i was 0.012 μM; with dihydrofolate the K_i was 0.11 μM as compared with 0.004 μM for MTX. Thus, lenghthening of the bridge by insertion of a CH_2 group between N^{10} and the phenyl ring brought about a 25-fold decrease in DHFR inhibition similar to the effect noted with (IV.50).

Compound (IV.51) was tested also for antibacterial activity and the ability to inhibit tumor cell growth *in vitro*. Activity against *L. casei* was extremely high (IC_{50} = 1.4 nM), though lower than that of AMT (IC_{50} = 0.006 nM). Against *S. faecium* the IC_{50} values of (IV.51) and AMT were 1.6 and 0.09 nM. Activity against tumour cells was much lower. HeLa cell growth was 50% inhibited in the presence of 10 μM (IV.51), while S180 murine sarcoma cell growth was 50% inhibited only when the drug concentration was 50 μM. Inhibition in HeLa cells was prevented by 100 μM folinic acid. Uptake experiments in the S180 cells revealed temperature-dependent exponential kinetics consistent with carrier-mediated active transport; however, detailed kinetic analysis was not performed. *In vivo* antitumour data were not reported.

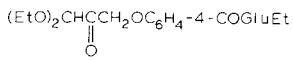
Replacement of the bridge N by other heteroatoms

2,4-Diamino compounds

The first work in this area was the synthesis of 10-oxa-10-deazaAMT (IV.52), which was reported as early as 1954 by Fairburn and co-workers [107]. The key building block in this synthesis was the α-ketoaldehyde diethyl acetal, (IV.54), which on reaction with 2,4,5,6-tetraaminopyrimidine at pH 3.0 (90 °C for 2 h) and subsequent vigorous treatment with HCl (100 °C, 15 min) afforded the diester (IV.53) in low yield. Compound (IV.54) was prepared from diethyl *N*-(4-hydroxybenzoyl)-L-glutamate by reaction with 2,3-epoxy-1,1-diethoxypropane followed by oxidation of the resultant secondary alcohol with chromic acid. Alkaline hydrolysis of (IV.53) yielded (IV.52). The product was assumed to be 6- rather than 7-substituted on the basis that the 5-amino group in 2,4,5,6-tetraaminopyrimidine is the most nucleophilic and therefore most likely to react with the keto group in (IV.54). Antibacterial activity against *S. faecium* was observed.

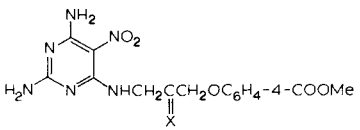


(IV.52) R = H
(IV.53) R = Et

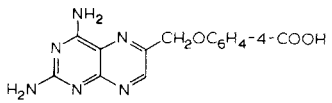


(IV.54)

In 1976, Nair and Campbell [108] devised a new and unambiguous route to (IV.52) that allowed more definitive conclusions to be made about whether N^{10} is essential for biological activity. The key intermediate in this synthesis was the nitro ketone (IV.55), which was generated from the nitro oxime (IV.56) on mild treatment with acid. Compound (IV.55) was obtained from methyl 4-hydroxybenzoate and 1-bromo-3-(*N*-phthalimido)acetone via a four step sequence culminating in the reaction of 1-amino-3-[(4-carbomethoxy)-phenoxy]-2-oximinopropane and 2,4-diamino-6-chloro-5-nitropyrimidine. Reduction of the nitro group in (IV.55) with sodium dithionite in aqueous DMF, ring closure in the presence of pyridine hydrochloride, and gentle oxidation in alkaline DMSO led to the 4-amino-4-deoxypteroic acid analogue (IV.57). Mixed anhydride condensation of (IV.57) with α-benzyl L-glutamate γ-linked to Merrifield resin, followed by alkaline hydrolysis, afforded (IV.52) in 85% yield (based on the amount of (IV.57) consumed). The UV absorption spectrum of the product [λ_{max} (0.1 M NaOH) 262 nm (ε 42,115), 371 (8,158); λ_{max} (0.1 M HCl) 248, 335 nm] was consistent with a 6-substituted 2,4-diaminopteridine structure. Potent antibacterial activity was found against both *L. casei* (IC_{50} = 0.11 nM) and *S. faecium* (IC_{50} = 11 nM). In agreement with historical experience with other classical antifolates, *L. casei* was the more sensitive organism.



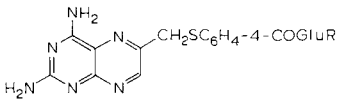
(IV.55) Y = O
(IV.56) Y = NOH



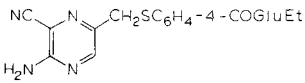
(IV.57)

When the identical reaction sequence was carried out with methyl 4-mercaptobenzoate instead of methyl 4-hydroxybenzoate, the product was 10-deaza-10-thioAMT (IV.58) [109]. The UV spectrum of (IV.58) [λ_{max} (0.1 M NaOH) 262 nm (ε 30,115), 285 (17,868), 377 (7,814); λ_{max} (0.1 M HCl) 248 nm (ε 24,723), 283 (17,283), 341 (10,648)] was similar to that of (IV.52) except for the presence of an extra maximum in the 280–290 nm region, presumably attributable to the sulphur atom on the phenyl ring. Compound (IV.58) was also prepared independently [110] by a sequence based on the Taylor pteridine synthesis. It should be noted that this reaction scheme, like other examples of this process involving a pre-existing *p*-aminobenzoylglutamate moiety, may lead to some degree of side-chain racemization. Diethyl L-glutamate, was condensed with diethyl 4,4′-dithiobis(benzoate) in the

presence of DCC, and the resulting disulphide was reduced with sodium borohydride to diethyl *N*-(4-mercaptobenzoyl)-L-glutamate, which was added directly to 2-amino-5-chloromethylpyrazine-3-carbonitrile to obtain the amino nitrile (IV.60) (47%). Annulation with guanidine then gave the diester (IV.59) (58%), which on alkaline hydrolysis (1.5 h in 3:1 mixture of 0.02 M NaOH and MeCN at room temperature) was converted to (IV.58) (96%). The NMR spectrum of (IV.58) in trifluoroacetic acid, showed the CH_2S protons as a singlet at δ 4.25, the phenyl protons as a pair of doublets (A_2B_2 quartet) at δ 7.15 and 7.45, and the C-7 proton as a singlet at δ 8.68. The relatively high downfield location of the C-7 proton was consistent with protonation of the pteridine moiety by trifluoroacetic acid. The UV spectrum [λ_{max} (0.01 M NaOH) 260 nm (ε 33,000), 280 (22,000), 373 (8,500)] was similar to, but not identical with, the one obtained by Nair *et al.* [109] in 0.1 M NaOH.



(IV.58) R = H
(IV.59) R = Et

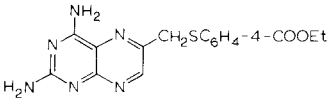


(IV.60)

10-Deaza-10-thiaAMT (IV.58) proved to be an excellent inhibitor of purified DHFR from *L. casei* [109]. Although a more relevant compound for comparison would have been AMT, it was clear that replacement of N^{10} by S was well tolerated, at least by this bacterial enzyme. Growth inhibitory potency against *L. casei* and *S. faecium* was compared to that of the 10-oxa analogue (IV.52), and was found to follow an interesting pattern. The potency of (IV.58) against *L. casei* (IC_{50} = 0.022 nM) was 5-fold greater than that of (IV.52), whereas potency against *S. faecium* (IC_{50} = 0.88 nM) was increased 12-fold. It thus appeared that while replacement of N^{10} by S was superior to replacement of N^{10} by O with both organisms, the magnitude of the change was greater with *S. faecium*. The results reinforced the important concept that cross-species structure–activity relationships among classical antifolates are subtle and not always quantitatively predictable.

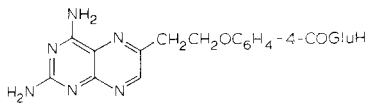
10-Deaza-10-thiaAMT (IV.58) and its diethyl ester (IV.59) were compared with MTX for their ability to inhibit DHFR and TS from *L. casei* [110]. Whereas the IC_{50} value of MTX in the DHFR assay was 3.3 nM, the IC_{50} values of (IV.58) and (IV.59) were 0.016 and 6 μM, respectively. Thus, substitution of S for the NMe group in MTX led to only a 5-fold decrease in DHFR binding, whereas esterification resulted in a 350-fold decrease. TS was not inhibited by either (IV.58) or (IV.59) at concentrations of up to 100 μM.

10-Deaza-10-thiaAMT (IV.58) was also compared with AMT as an inhibitor of the growth of MTX-sensitive and MTX-resistant *S. faecium* and *L. casei* [110]. The potency of (IV.58) against MTX-sensitive *S. faecium* (5.2 nM) was slightly lower than that of AMT (2.3 nM), whereas against MTX-sensitive *L. casei* the converse was true. It is interesting that in these experiments MTX was 6-fold more potent than AMT against *S. faecium* but only 3-fold more potent against *L. casei.* This underscored again the importance of selecting an appropriate standard when making structure–activity correlations among classical antifolates. With some compounds such as (IV.58) this choice can be difficult. An interesting feature of the lipophilic diester (IV.60) in comparison with the parent acid (IV.58) was that the diester was nearly 20-times more potent than MTX against MTX-resistant *S. faecium.* Somewhat surprisingly, the corresponding ester (IV.61), without an amino-acid side-chain, was also more active than MTX. The mechanism by which this lipophilic ester overcomes MTX resistance in *S. faecium* has not been explored.

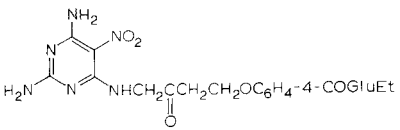


(IV.61)

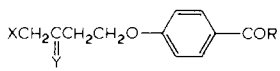
11-Deaza-11-oxahomoAMT (IV.62) was synthesized by Nair *et al.* [111] by a modification of their synthesis of 10-deaza-10-oxaAMT (IV.52). 1-Bromo-4-[4-(carbomethoxy)phenoxy]-2-butanone (IV.63) was converted successively to the azide (IV.64), the ketal (IV.65), and the acid (IV.66). Mixed anhydride coupling of (IV.66) and diethyl L-glutamate, followed by catalytic hydrogenation of the azido group, yielded the amine (IV.67), which, on addition to 2,4-diamino-6-chloro-5-nitropyrimidine and hydrolysis of the ketal group with acid, gave the key intermediate, (IV.68). Dithionite reduction led



(IV.62)



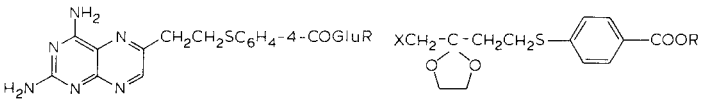
(IV.68)



(IV.63) X = Br, Y = O, R = OMe
(IV.64) X = N_3, Y = O, R = OMe
(IV.65) X = N_3, Y = —OCH_2CH_2O—, R = OMe
(IV.66) X = N_3, Y = —OCH_2CH_2O—, R = OH
(IV.67) X = NH_2, Y = —OCH_2CH_2O—,
R = NHCH(COOEt)CH_2CH_2COOEt

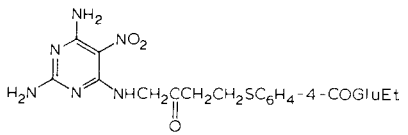
to spontaneous ring closure to a dihydropteridine, and ester hydrolysis and mild oxidation with DMSO or $KMnO_4$ finally yielded (IV.62) [λ_{max} (0.1 M NaOH) 259 nm (ε 29,204), 367 (5,416); λ_{max} (0.1 M HCl) 242 nm (ε 22,000), 335 (7,677)].

A similar approach was followed to obtain 11-deaza-11-thiahomoAMT (IV.69) [112]. Starting from 1-chloro-3-buten-2-one and methyl 4-mercaptobenzoate, the azido ketal (IV.71) was prepared in six steps in excellent overall yield. Mixed anhydride coupling of (IV.71) and diethyl L-glutamate followed by reduction of the azido group with sodium dithionite gave the amino ketal (IV.72). Condensation of (IV.72) with 2,4-diamino-6-chloro-5-nitropyrimidine and hydrolysis of the ketal gave the nitro ketone (IV.73), which on reduction with sodium dithionite, ring closure at pH 5, and aerobic oxidation, gave the diester (IV.70). Alkaline hydrolysis converted (IV.70) to (IV.69) [λ_{max} (0.1 M NaOH) 258 nm (ε 32,029) 372 (7,139); λ_{max} (0.1 M HCl) 248 nm (ε 26,944), 337 (9,829)]. The UV spectrum of (IV.69) was unlike that of (IV.58) in that, even though (IV.69) similarly contained a sulphur atom on the phenyl ring, an absorption maximum in the 280–290 nm region was absent. This spectral difference between 10-deaza-10-thiaAMT and 11-deaza-11-thiahomoAMT suggests that the additional CH_2 group in the bridge exerts some as yet unknown influence on the molecular conformation of the chromophore.



(IV.69) R = H
(IV.70) R = Et

(IV.71) X = N_3, R = OH
(IV.72) X = NH_2, R = NHCH(COOEt)CH_2CH_2COOEt



(IV.73)

The availability of the oxa and thia analogues of AMT and homoAMT allowed comprehensive evaluation of the enzyme inhibitory activity, cellular uptake, and antibacterial and antitumour activity of this series of bridge altered compounds [108–113].

In assays against purified DHFR from *L. casei*, 10-deaza-10-oxaAMT (IV.52), 10-deaza-10-thiaAMT (IV.58), and 10-deazaAMT (IV.91; *vide infra*) all showed an IC_{50} value of 4 nM, whereas the values observed with 11-deaza-

11-oxahomoAMT (IV.62) and 11-deaza-11-thiahomoAMT (IV.69) were 0.2 and 0.4 μM, respectively [111]. Thus, introduction of an extra bridge CH_2 group produced a 50- to 100-fold loss of DHFR inhibition, presumably because the α-carboxyl of the glutamate side-chain, which was now separated by a greater distance from the 2,4-diamino moiety, interacted less effectively with its binding site on the enzyme. It may be noted the IC_{50} value of MTX against *L. casei* DHFR was only 1 nM [112]. The homoAMT analogues IV.62 and IV.69 were also less potent than the AMT analogue IV.52 and IV.58 as inhibitors of *S. faecium* and *L. casei* growth in culture [111]. The thia compounds tended to be more potent than the oxa compounds in each instance, and *L. casei* was the more sensitive organism. The difference in growth inhibitory potency was considerably less than the difference in enzyme inhibitory potency.

Transport and growth inhibition studies were performed with the oxa and thia analogues of AMT in HeLa cells to assess their therapeutic potential in a mammalian *in vitro* system [113]. The IC_{50} value of the compounds as competitive inhibitors of the uptake of [^{3}H]MTX or [^{3}H]folate was determined. The concentration of labelled drug in each instance was 0.82 μM, and the experiment was performed in the absence of serum to minimize binding to serum proteins. The IC_{50} value of 10-deaza-10-oxaAMT (IV.52) as an inhibitor of MTX uptake was found to be 0.74 μM, whereas that of 10-deaza-10-thiaAMT (IV.58) was 2.7 μM. As inhibitors of the uptake of folic acid, on the other hand, (IV.52) and (IV.58) had IC_{50} values of 71 and 6 μM, respectively. It thus appeared that these compounds were more efficient substrates for the MTX than for the folate carrier system, and that with both carrier systems the oxa analogue was a better substrate than the thia analogue. This suggested that the oxa analogue ought to show the greater growth inhibitory activity against mammalian cells, in contrast to the bacterial assays, where the converse had been observed. In agreement with this expectation, when DNA synthesis and cell growth of the HeLa line were compared, (IV.52) again proved to be the more potent analogue. The IC_{50} value of (IV.52) as an inhibitor of [^{3}H]dUrd incorporation into DNA was 0.085 μM, as compared with 0.075 and 0.7 μM for MTX and (IV.58), respectively. Thus, (IV.52) was approximately equipotent with MTX in this assay, but was 5- to 10-fold less potent than (IV.58). When cell growth inhibitions were measured, the IC_{50} values for (IV.52), MTX, and (IV.58) were 0.075, 0.01 and 1.5 μM, respectively. These results clearly demonstrated O for N substitution to be more favourable than S for N substitution where growth inhibition of a mammalian cell line was concerned.

In an extension of the transport studies with (IV.52) and (IV.58), the ability

of the homoAMT analogues (IV.62) and (IV.69) to compete with [^{3}H]MTX transport in HeLa cells was examined [111]. The IC_{50} value of the oxa analogue (IV.62) was 21 μM, whereas that of (IV.69) was 25 μM. Thus, insertion of an extra CH_2 group in the bridge resulted in 30- and 10-fold decreases in MTX transport inhibition in the oxa and thia series, respectively.

Kinetic constants for the inhibition of MTX transport in L1210 leukaemia and Ehrlich carcinoma cells were determined for (IV.62) and (IV.69) in comparison with AMT [111]. The K_i of MTX itself as an inhibitor of [^{3}H]MTX transport was 3.7 μM in L1210 cells and 10 μM in Ehrlich cells, while that of AMT was 1.2 and 2 μM, respectively, indicating that AMT is transported somewhat more efficiently than MTX by these cell lines. The K_i values for (IV.62) and (IV.69) as inhibitors of MTX transport in L1210 cells were 23 and 24 μM, while the corresponding values in Ehrlich cells were 12 and 13 μM. Thus, insertion of an extra CH_2 group and replacement of N by O or S resulted in a 6-fold decrease in affinity for the MTX transport system in L1210 cells but had little effect in Ehrlich cells. As with other biological parameters, therefore, quantitative structure–activity correlations based on the transport of these compounds tended to vary depending on the cell line used.

In another study, the DHFR binding and membrane transport properties of 10-deaza-10-oxaAMT (IV.52) were studied in comparison with AMT by Sirotnak *et al.* [13]. The K_i of (IV.52) as an inhibitor of DHFR from L1210 cells was found to be 280 nM as compared with 0.0032 nM for AMT. Thus, (IV.52) was much less potent against the mammalian enzyme than Nair *et al.* [111] had found it to be against *L. casei* enzyme. The ability of (IV.52) to inhibit [^{3}H]MTX transport was virtually the same as that of unlabelled MTX and only 3-fold lower than that of AMT. Despite the 90,000-fold difference in anti-DHFR activity between (IV.52) and AMT and the likelihood that the two compounds are transported at similar rates across the cell membrane, the IC_{50} value of (IV.52) against L1210 cells proved only 2-fold lower than that of AMT. The authors concluded that certain compounds such as (IV.52) exhibit 'analogue specific aberrancy', i.e., the growth-inhibitory activity of these compounds is for some reason much greater than would be expected from spectrophotometric DHFR inhibition data and/or transport data alone. For such 'aberrant' antifolates, it was argued, an indirect assay measuring DHFR inhibition *in situ* would be a better predictor of growth inhibition.

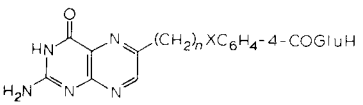
In vivo testing of the oxa and thia analogues of AMT and homoAMT has been carried out against L1210 murine leukaemia [111]. The most active member of the group was 10-deaza-10-oxaAMT (IV.52), which gave a 179% ILS when given at a dose of 3 mg/kg (qd × 9), and a 125% ILS at 0.38 mg/kg.

The potency and therapeutic efficacy of IV.52 compared favourably with those of 10-deazaAMT (IV.91, *vide infra*) (172% ILS at 12 mg/kg, qd × 9) and isoAMT (IV.152, *vide infra*) (132% ILS at 4 mg/kg, qd × 9). 11-Deaza-11-oxahomoAMT (IV.62) was less active, with an ILS of only 85–90% over a nearly 10-fold range of doses from 0.38 to 3.0 mg/kg (qd × 9). The strikingly shallow dose–response curve with (IV.58) and to a lesser extent (IV.52) was suggestive of a high margin of safety. 10-Deaza-10-thiaAMT (IV.58) was nearly as effective as its 10-oxa counterpart (IV.52) in prolonging survival, but was less potent on a molar basis, giving a 130% ILS at 48 mg/kg (qd × 9). Interestingly, while one might have expected on the basis of structural analogy that 11-deaza-11-thiahomoAMT (IV.69) would be less potent than the 11-oxa analogue, (IV.62), this did not prove to be correct. In fact, (IV.69) had about the same activity as (IV.51), giving a 95–105% ILS over a range of doses from 0.38 to 3.0 mg/kg. Overall, it appears that the potency of (IV.52), (IV.58), and (IV.69) against L1210 leukaemia in mice is similar to that of AMT, whereas (IV.62), for reasons not understood at present, more closely resembles MTX.

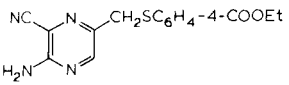
*2-Amino-4-(3*H*)-oxo compounds*

10-Deaza-10-oxafolic acid (IV.74) [108], 10-deaza-10-thiafolic acid (IV.75) [114], 11-deaza-11-oxahomofolic acid (IV.76) [115], and 11-deaza-11-thiahomofolic (IV.77) [116] have all been synthesized by procedures generally analogous to those yielding the corresponding 2,4-diamines. Compound (IV.75) has also been obtained by the following alternative route [110] based on the Taylor pteridine synthesis: 2-amino-5-chloromethyl-pyrazine-3-carbonitrile was condensed with ethyl 4-mercaptobenzoate (freshly prepared by sodium borohydride reduction of the corresponding disulphide), the resulting amino nitrile (IV.78) (90%) was cyclized to ethyl 4-amino-4-deoxy-10-deaza-10-thiapterote (IV.61) (67%) with guanidine, and the ester and 4-amino group were cleaved by vigorous alkaline hydrolysis (2 M NaOH in refluxing EtOH for 5 h) to obtain the acid (IV.79) (71%); mixed anhydride coupling to diethyl L-glutamate followed by alkaline hydrolysis then yielded (IV.75). The NMR spectrum of (IV.75) in trifluoroacetic acid [110] showed the CH_2S protons as a singlet at δ 4;46, the phenyl protons as a pair of doublets at δ 7.37 and 7.67, and the C-7 protons as a singlet at δ 8.9. The NMR spectrum in NaOD-D_2O [114] showed the C-7 proton as a singlet at δ 8.54, suggesting that the chemical shift of this proton is dependent on whether the adjoining nitrogen is protonated. The UV spectrum [λ_{max} (0.01 M NaOH) 257 nm (ε 34,000), 280 (22,700), 365 (9,350) [110]; λ_{max} (0.1 M NaOH) 261 nm (ε 32,850), 285sh (21,068), 369 (9,368) [114]], showing a small but noticeable bathochromic displacement at the higher base concentration, was consistent with the assigned structure, and

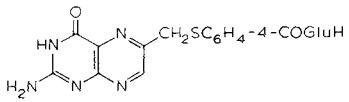
was very similar to the spectrum of (IV.77) [116]. The NMR spectrum of (IV.76) in trifluoroacetic acid [115] showed the CH_2CH_2O protons as a triplet of triplets centered at δ 4.6, the phenyl protons as a pair of doublets at δ 7.05 and 7.83, and the C-7 proton as a singlet at δ 8.95. The UV spectrum [λ_{max} (0.1 M NaOH) 253 nm (ε 37,027), 362 (6,976)], which resembled that of (IV.74), was consistent with the assigned structure [115] and was notable for its lack of phenylthioether-related absorption at 280–290 nm (cf. the spectrum of (IV.75)). The NMR spectrum of (IV.77) in trifluoroacetic acid [116] showed the CH_2CH_2S protons as a broad signal centered at δ 3.55, the phenyl protons as a pair of doublets at δ 7.45 and 7.8, and the C-7 proton as a singlet at δ 8.80 (compare the more upfield location of all the corresponding protons in (IV.76)). The UV spectrum of (IV.77) [λ_{max} (0.1 M NaOH) 256 nm (ε 29,389), 275sh (19,288), 366 (7,888); λ_{max} (0.1 M HCl) 255 nm (ε 15,151), 290 (14,911)] was consistent with expectations based on the spectra of (IV.74) and (IV.75). Reduction of (IV.76) with sodium dithionite afforded the 7,8-dihydro derivative (IV.80), while catalytic hydrogenation gave a mixture of the 'natural' and 'unnatural' diastereomers of the 5,6,7,8-tetrahydro compound (IV.82). Similar reductions of (IV.77) afforded the 11-thia analogues (IV.81) [λ_{max} (0.1 M NaOH) 280, 320 nm] and (IV.76) [λ_{max} (pH 7.6) 290 nm]. The 'natural' diastereomers of (IV.82) and (IV.83) were prepared from (IV.80) and (IV.81) by reduction with *L. casei* DHFR.



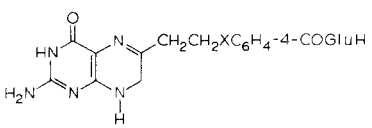
(IV.74) n = 1, X = O
(IV.75) n = 1, X = S
(IV.76) n = 2, X = O
(IV.77) n = 2, X = S



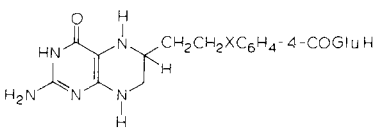
(IV.78)



(IV.79)



(IV.80) X = O
(IV.81) X = S



(IV.82) X = O
(IV.83) X = S

The ability of 10-deaza-10-thiafolic acid (IV.75) to inhibit purified DHFR (IC_{50} = 50 μM) and TS (IC_{50} > 30 μM) from *L. casei* has been found to be

low [110, 114]. In growth-inhibition assays against *S. faecium* and *L. casei* in the presence of 2 nM folic acid, IC_{50} values of 110 and 1,600 nM, respectively, were obtained [110]. In other experiments [114], (IV.75) had an IC_{50} of 2 nM against *S. faecium* in the presence of 0.6 nM folic acid, while against *L. casei* in the presence of 0.12 nM folic acid the IC_{50} was 17 nM. Thus, antibacterial potency was dependent on the folate content of the growth medium. Growth inhibition data against mammalian cells were not reported.

The IC_{50} values of the 11-deaza-11-oxahomofolic acid (IV.76) against *S. faecium* and *L. casei* were found to be 31 and 700 nM, respectively, while the corresponding values for MTX were 11 and 15 nM [115]. Thus, the homofolate analogue was only one-third less potent than MTX against *S. faecium* but 45-fold less potent against *L. casei*, showing the latter organism to be much more sensitive to the structural changes embodied in this compound. The 7,8-dihydro derivative, (IV.80), had an IC_{50} value similar to (IV.76), while the tetrahydro derivative (IV.82) in the chemically reduced (6-*R,S*)-L-form (i.e., a mixture of 'natural' and 'unnatural' diastereomers) was much more active, with an IC_{50} of 1.5 nM against *S. faecium* and 22 nM against *L. casei*. The enzymatically synthesized 'natural' diastereomer (6*S*)-L-(IV.82) was 5- to 10-fold less active than (6-*R,S*)-L-(IV.82). This suggested that the active component in (6-*R,S*)-L-(IV.82) was actually the 'unnatural' diastereomer, (6*R*)-L-(IV.82). The same surprising observation was made earlier in studies on the 'natural' and 'unnatural' diastereomers of H_4HFA (IV.27) [83]. An important difference between (6-*R,S*)-L-(IV.82) and (6-*R,S*)-L-(IV.27), however, was that the 'natural' diastereomer of H_4HFA supported bacterial growth, while that of (IV.82) apparently did not. A striking result observed with (6-*R,S*)-L-(IV.82) and to a lesser degree with (IV.76) and (IV.80) was low cross-resistance with MTX in assays against MTX resistant *L. casei*. While the IC_{50} of MTX against this organism was $> 100\ \mu M$ ($> 10^6$-fold resistance), the IC_{50} of (6-*R,S*)-L-(IV.82) was only 42 nM (28-fold resistance). In an attempt to gain insight into the mechanism of action of (IV.76), (IV.80), and (IV.82), these compounds were tested as inhibitors of purified TS from *L. casei*, but surprisingly none of them inhibited the enzyme at concentrations below $10\ \mu M$. Thus growth inhibition appeared to involve a folate-dependent process other than dTMP synthesis. In all likelihood, given what is now known about the action of homofolates, this relates to purine synthesis.

In antibacterial assays against *L. casei* and *S. faecium*, the 11-thia analogue (IV.77) was not inhibitory at concentrations below $3\ \mu M$, was neither a substrate nor an inhibitor of *L. casei* DHFR, and was not an inhibitor of *L. casei* TS [116]. Replacement of CH_2CH_2O by CH_2CH_2S in the 2-amino-4(3*H*)-oxo series therefore led to severe loss of antibacterial activity. How-

ever, the chemically reduced tetrahydro derivative (6-*R,S*)-L-(IV.83) was still a potent inhibitor of *S. faecium* (IC_{50} = 2.2 nM), with activity comparable to H_4HFA. Interestingly, (6-*R,S*)-L-(IV.83) was > 600-fold less potent (IC_{50} = 1400 nM) against *L. casei* than against *S. faecium*, demonstrating that replacement of CH_2CH_2O by CH_2CH_2S in the tetrahydro series enhanced activity against one of these organisms while decreasing it against the other.

The availability of (IV.74) and (IV.75) made it of interest to determine how replacement of N^{10} by S in folic acid would affect substrate activity relative to folylpolyglutamate synthetase [94]. Inhibition of this enzyme could in theory be the basis for a therapeutic strategy aimed at blocking polyglutamylation of endogenous cellular folates, thereby interfering with folate accumulation and inducing a folate deficient state. In comparative assays using partially purified enzyme from mouse liver, folic acid had an apparent K_m of 140 ± 47 μM, while for 10-deaza-10-thiafolic acid (IV.75) this value was 88 ± 40 μM. Thus, replacement of N by S increased binding to the enzyme. However, the V_{max} was decreased by about 50%, so that the $V_{max}/K_{m(app)}$ ratio for the two compounds was essentially the same. Whether folylpolyglutamate synthetase in intact cells can add more than one glutamate to (IV.75), which would obviously be relevant to biological action *in vivo*, has not yet been determined. Comparisons of the substrate activities of the dihydro and tetrahydro derivatives would also be of interest.

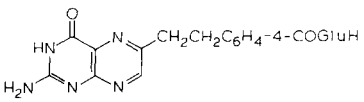
The potent growth inhibitory activity displayed by 10-deaza-10-oxafolic acid (IV.74) and 10-deaza-10-thiafolic acid (IV.75) against bacteria unfortunately has not been found to extend to mammalian cells [114]. Against cultured HeLa cells, for example, the IC_{50} values of both compounds were > 100 μM. This was in marked contrast to the IC_{50} values of the corresponding 2,4-diamines, which were 0.075 and 1.5 μM. Predictably, (IV.74) and (IV.75) were also much less active than the 2,4-diamino analogues as inhibitors of [^{14}C]deoxyuridine incorporation into DNA. These results clearly showed that, while replacement of N^{10} in folic acid by O or S interferes with folic acid utilization in bacteria, it does this much less efficiently in mammalian cells. It should be noted, on the other hand, that the growth inhibition experiments with HeLa cells were performed in medium stated to contain 2.2 μM folic acid, a concentration that could interfere with the ability of the cells to take up drug if a common pathway for membrane transport is shared. Evidence that (IV.74) and (IV.75) are in fact taken up by the same pathway as folic acid was obtained in competitive transport experiments using [^{3}H]folic acid and [^{3}H]MTX. Uptake of 0.8 μM [^{3}H]folic acid was inhibited by 50% with either 7.2 μM (IV.74) or 7.2 μM (IV.75), whereas uptake of [^{3}H]MTX under the

same conditions was only minimally affected. From this it appears possible that the low potency of (IV.74) and (IV.75) in the HeLa cell growth assay was due to the relatively high concentration of folic acid in the medium.

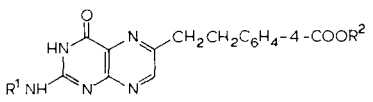
Replacement of N^{10} by CHR or CH_2CHR

Chemical synthesis

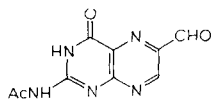
10-Deazafolic acid (IV.84) was first synthesized in 1971 [117] by a scheme starting from 2-acetamido-4(3*H*)-oxopteridine-6-carboxaldehyde (IV.85). Condensation of the aldehyde with the ylide formed from (4-ethoxycarbonylbenzyl)triphenylphosphonium bromide and NaOMe in DMF gave a 79% yield of the ester, (IV.86). Catalytic hydrogenation of the 9,10-double bond was accompanied by 7,8-reduction, but the aromatic pyrazine ring could readily by regenerated by treatment with H_2O_2. Simultaneous ester and amide hydrolysis with KOH, followed by reacetylation of the 2-amino group with acetic anhydride, afforded N^2-acetyl-10-deazapteroic acid (IV.87) in approximately 30% yield starting from the olefin ester (IV.86). Condensation of (IV.87) with diethyl L-glutamate in pyridine in the presence of DCC and subsequent hydrolysis with 0.1 M KOH led to (IV.84). The yield in the DCC reaction was relatively low. The UV spectrum [λ_{max} (pH 13) 253 nm (ε 31,300), 363 (7,500)] was consistent with replacement of N by C at position 10. The NMR spectrum in trifluoroacetic acid showed the bridge CH_2CH_2 protons as a multiplet at δ 3.4, the phenyl protons as a pair of A_2B_2 doublets at δ 7.40 and 7.85, and the C-7 proton as a singlet at δ 8.7. Alkaline hydrolysis of the olefin ester (IV.86), followed by N^2-reacetylation, DCC-mediated coupling to diethyl L-glutamate, and cleavage of the ester groups with 0.1 M KOH afforded 9,10-dehydro-10-deazafolic acid (IV.90).



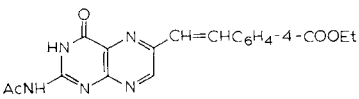
(IV.84)



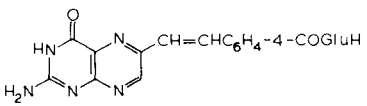
(IV.87) R^1 = MeCO, R^2 = H
(IV.88) R^1 = Me_3CCO, R^2 = *t*-Bu
(IV.89) R^1 = Me_3CCO, R^2 = H



(IV.85)

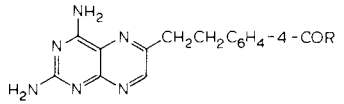


(IV.86)

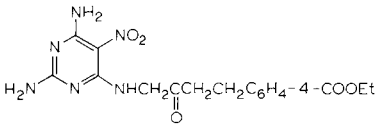


(IV.90)

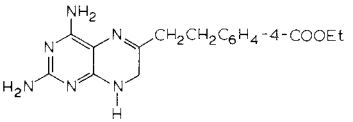
The earliest member of the 2,4-diamino series, 10-deazaAMT (IV.91), was prepared in 1974 by DeGraw *et al.* [118] by a mixed anhydride reaction in DMSO solution between 4-amino-4-deoxy-10-deazapteroic acid (IV.92) and either *N,O,O*-trimethylsilylated L-glutamic acid or γ-benzyl L-glutamate α-linked to Merrifield resin. Upon completion of the coupling reaction, ester groups were cleaved with base to give the diacid (IV.91) [λ_{max} (pH 13) 256 nm (ε 32,500), 372 (7,475); λ_{max} (pH 1) 243 (ε 26,800), 340 (8,800)]. The acid (IV.92) was synthesized [112] from the nitro ketone (IV.94) by reduction of the nitro group with Zn, reoxidation of the spontaneously formed dihydropteridine (IV.95) with 30% H_2O_2, and ester hydrolysis with 5% NaOH in 2-methoxyethanol (100 °C, 30 min). The nitro ketone (IV.94) was obtained by condensing the amino semicarbazone (IV.96) with 2,4-diamino-6-chloro-5-nitropyrimidine and removing the semicarbazone group with 90% trifluoroacetic acid.



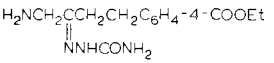
(IV.91) R = NHCH(COOH)CH_2CH_2COOH
(IV.92) R = OH
(IV.93) R = OBu-*t*



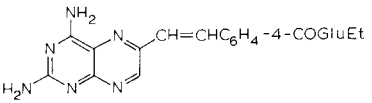
(IV.94)



(IV.95)



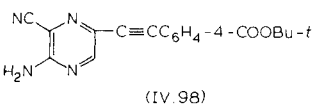
(IV.96)



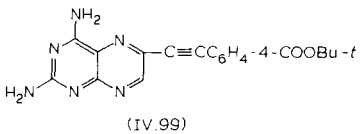
(IV.97)

An alternative method of synthesis of (IV.91) from 2,4-diamino-6-bromomethylpteridine was described in 1980 by Piper and Montgomery [120]. The bromo compound was treated with triphenylphosphine in DMA (60–63 °C, 1.5 h), and the resultant ylide was condensed with diethyl *N*-(4-formylbenzoyl)-L-glutamate to obtain diester (IV.97) in 78% yield. Catalytic reduction of the 9,10 double bond resulted in partial reduction of the pteridine ring to a 7,8-dihydro derivative. Reoxidation of ring B with H_2O_2 followed by ester hydrolysis with NaOH afforded (IV.91) (62%).

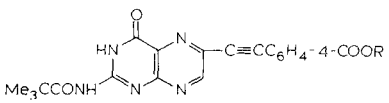
An ingenious new pteridine synthesis was very recently employed by Taylor and Ray [121] to prepare 4-amino-4-deoxy-10-deazapteroic acid (IV.92), thus providing an alternative route to (IV.91). The key step in this sequence was the formation of *t*-butyl 4-[(2-amino-3-cyanopyrazin-5-yl)ethynyl]-benzoate ((IV.98), 48% yield) from 2-amino-5-bromopyrazine-3-carbonitrile [122] and *t*-butyl 4-ethynylbenzoate in the presence of Et_3N and catalytic amounts of $PdCl_2$-Ph_3P and CuI. Annulation with guanidine in refluxing *t*-butanol converted (IV.98) to the ester (IV.99) (87%), which on catalytic hydrogenation followed by acidolysis with HCl in nitromethane afforded successively the ester (IV.93) (72%) and acid (IV.92) (87%). By means of this approach, it was also possible to obtain N^2-pivaloyl-10-deazapteroic acid (IV.87), a useful precursor to 10-deazafolic acid (IV.84). In this instance, 2-(*N*-pivaloyl)amino-6-chloropteridin-4(3*H*)-one [122] was condensed with *t*-butyl 4-ethynylbenzoate in the presence of Et_3N, $Pd(OAc)_2$-(*p*-$MeC_6H_4)_3P$, and CuI to obtain the acetylenic ester, (IV.101) (54%). Catalytic hydrogenation gave a 7,8-dihydro derivative, which was readily reoxidized with oxygen and then selectively hydrolyzed with HCl in nitromethane at 0 °C to form (IV.89) (60% combined yield). The N^2-pivaloyl group proved especially useful in this synthesis because of its solubilizing properties and the relatively mild conditions under which it could ultimately be removed. Selective ester cleavage to form (IV.100) from (IV.101) should be possible but was not reported.



(IV.98)



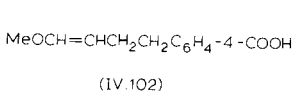
(IV.99)



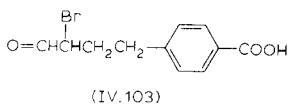
(IV.100) R = H
(IV.101) R = Bu-*t*

Because the original synthesis of 10-deazaAMT (IV.91) via the Boon-Leigh approach had given an overall yield of only 5%, DeGraw *et al.* [123] subsequently developed also a modified Waller-type scheme that not only proceeded in somewhat better overall yield but was amenable to the preparation of 10-alkyl-10-deaza derivatives. Condensation of 3-methoxyallyl chloride *in situ* with the dianion of *p*-toluic acid (generated with 2 equivalents of lithium diisopropylamide in cold THF) yielded the enol ether (IV.102). On bromination and mild acid hydrolysis at pH 1–2, (IV.102) gave the α-bromoalde-

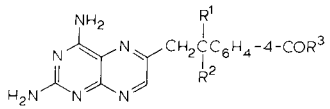
hyde (IV.103) in excellent yield. Condensation of (IV.103) with 2,4,5,6-tetraaminopyrimidine in aqueous acetic acid (45–50 °C, 1.5 h) and direct oxidation of the resultant dihydropteridine with KI_3 led to (IV.92) (12%). Further elaboration of the glutamate side-chain was carried out by mixed anhydride coupling and alkaline hydrolysis as described earlier [118]. A similar sequence starting from 4-ethylbenzoic acid, 4-propylbenzoic acid, and 4-isopropylbenzoic yielded the 10-deazapteroic acid analogues (IV.104) (32%), (IV.105) (32%), and (IV.106) (13%), from which were then prepared the 10-alkyl-10-deaza analogues (IV.107) (38%), (IV.108) (30%), and (IV.109) (26%).



(IV.102)



(IV.103)

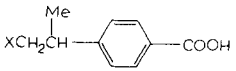


(IV.104) R^1 = Me, R^2 = H, R^3 = OH
(IV.105) R^1 = Et, R^2 = H, R^3 = OH
(IV.106) R^1 = R^2 = Me, R^3 = OH
(IV.107) R^1 = Me, R^2 = H, R^3 = NHCH(COOH)CH_2CH_2COOH
(IV.108) R^1 = Et, R^2 = H, R^3 = NHCH(COOH)CH_2CH_2COOH
(IV.109) R^1 = R^2 = Me, R^3 = NHCH(COOH)CH_2CH_2COOH

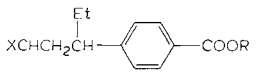
More recently, DeGraw *et al.* [124] returned to, and improved, the Boon-Leigh approach to obtain the 10-methyl and 10-ethyl analogues (IV.107) and (IV.108) with C^{10} in either the racemic (*rac*,L-(IV.107), *rac*,L-(IV.108)) or enantiomerically pure form (*d*,L-(IV.107), *l*,L-IV.107, *d*,L-IV.108, *l*,L-IV.108). 4-Ethoxycarbonylacetophenone was condensed with benzyl (dimethylphosphono)acetate in the presence of NaH (60% yield), and the resultant α,β-unsaturated ester was catalytically reduced and debenzylated to *d,l*-(IV.110) (89% yield). Resolution was accomplished at this stage via the diastereomeric quinine salts. Sequential treatment with thionyl chloride, diazomethane, and HCl gave the α-chloroketone *d,l*-(IV.111), *d*-(IV.111), or *l*-(IV.111). The chloroketone was converted to the racemic or optically pure α-azidoketone (IV.112) (90% yield) by reaction with NaN_3 and to the racemic or optically pure α-aminoketone (IV.113) (85% yield) by catalytic hydrogenation. The semicarbazone derivative of (IV.113) was condensed with 2,4-diamino-6-chloro-5-nitropyrimidine in DMF in the presence of 2,4,6-trimethylpyridine as an acid scavenger, the semicarbazone group was removed with trifluoroacetic acid, the nitro group was reduced with Zn dust in hot acetic acid, the resultant dihydropteridine was reoxidized *in situ* with H_2O_2 and the ester group hydrolyzed with base to obtain racemic or enantiomerically pure (IV.104). Attachment of the L-glutamate moiety via mixed anhydride coupling

and alkaline hydrolysis were carried out in the usual way to obtain *rac*,L-(IV.107), *d*,L-(IV.107), or *l*,L-(IV.107).

For the preparation of the 10-ethyl-10-deaza analogues *rac*,L-(IV.108), *d*,L-(IV.108), and *l*,L-(IV.108), a somewhat different scheme was followed to form the required racemic or optically active acid ester (IV.114) [124]. The dilithium salt of 4-*n*-propylbenzoic acid was condensed with allyl bromide, and the resulting acid was esterified with MeOH-HCl to obtain the olefinic ester, (IV.115). On oxidation with RuO_2-$NaIO_4$, (IV.115) yielded (IV.114) (77%). Resolution of (IV.114) was achieved efficiently with *d*-(1-phenylethyl)amine. The remaining steps from (IV.114) to (IV.108) were the same as those used to form the 10-methyl-10-deaza analogue, (IV.107).

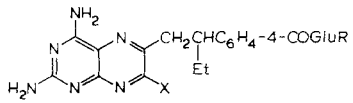


(IV.110) X = COOH
(IV.111) X = $COCH_2Cl$
(IV.112) X = $COCH_2N_3$
(IV.113) X = $COCH_2NH_2$



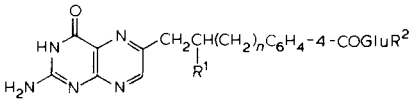
(IV.114) X = HOOC, R = Me
(IV.115) X = CH_2=CH, R = Me

A novel method of 7-hydroxylation of 10-ethyl-10-deazaAMT (IV.108) has recently been described [125]. The dimethyl ester (IV.117) was treated with a large excess of diethyl phosphorocyanidate and Et_3N in aqueous *N*-methylpyrrolidin-2-one at 75 °C for 19 h to obtain the 7-cyano derivative (IV.118) (59% yield). The presence of the cyano group in (IV.118) was not discernable in the infrared spectrum, but was inferred from a marked bathchromic shift in the UV spectrum [λ_{max} (95% EtOH) 224 sh (log ε = 4.34), 236 sh (4.32), 277 (4.32), 413 (3.74)] and the absence of a peak in the NMR spectrum corresponding to the C-7 proton, which was visible in the starting ester (IV.117) at δ 8.32 in d_6-DMSO solution. Alkaline hydrolysis of the ester groups in (IV.118) was accompanied by displacement of the 7-cyano substituent to form (IV.116) (49% yield). The same process was also used to convert dimethyl MTX to 7-hydroxyMTX, and may be general for pteridine antifolates. Although the precise mechanism of this Reissert-like reaction is unknown, it is important to note that addition does not occur unless water is present.



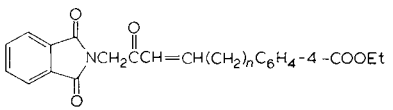
(IV.116) X = OH, R = H
(IV.117) X = H, R = Me
(IV.118) X = CN, R = Me

A general route to the 10-deazafolates and 11-deazahomofolates (IV.119)–(IV.123) was recently developed by Nair *et al.* [126]. This method should be applicable also to the synthesis of the corresponding 2,4-diamines. Key intermediates for the synthesis of these compounds were 1-(4-methoxycarbonylphenyl)-4-phthalimido-1-buten-3-one (IV.129) and either 1-(4-methoxycarbonylphenyl)-5-phthalimido-2-penten-4-one (IV.130) or 1-(4-methoxycarbonylphenyl)-5-phthalimido-1-penten-4-one (IV.131). Reduction of these unsaturated ketones with zinc in acetic acid gave the alcohols (IV.132) and (IV.133), while conjugate additions of Grignard reagents led to the alcohols (IV.134)–(IV.136). On reoxidation with chromic acid, condensation with hydroxylamine, and hydrazinolysis of the phthalimide group, the alcohols (IV.132)–(IV.136) were converted to the amino oxime esters (IV.137)–(IV.141). The latter were then condensed with 2-amino-4-chloro-5-nitro-6(1*H*)-oxopyrimidine to obtain the intermediates (IV.142)–(IV.146). Removal of the oxime group with trifluoroacetic acid, reduction of the nitro group with sodium dithionite (ring closure to a 7,8-dihydropteridine), and treatment with alkaline $KMnO_4$ (ester cleavage and oxidation of the 7,8-dihydropteridine) afforded the 10-deazapteroic and 11-deazahomopteroic acid analogues (IV.147)–(IV.151). Mixed anhydride coupling to diethyl L-glutamate followed by alkaline hydrolysis of esters (IV.124)–(IV.128) then gave the desired products (IV.119)–(IV.123).

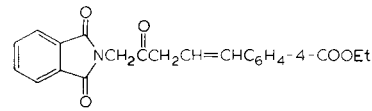


(IV.119) n = 0, R^1 = R^2 = H
(IV.120) n = 1, R^1 = R^2 = H
(IV.121) n = 0, R^1 = Me, R^2 = H
(IV.122) n = 0, R^1 = Et, R^2 = H
(IV.123) n = 1, R^1 = Me, R^2 = H

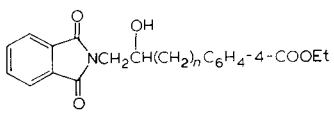
(IV.124) n = 0, R^1 = H, R^2 = Et
(IV.125) n = 1, R^1 = H, R^2 = Et
(IV.126) n = 0, R^1 = Me, R^2 = Et
(IV.127) n = 0, R^1 = R^2 = Et
(IV.128) n = 1, R^1 = Me, R^2 = Et



(IV.129) n = 0
(IV.130) n = 1



(IV.131)



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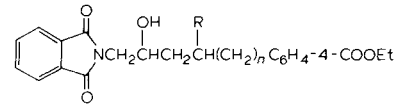

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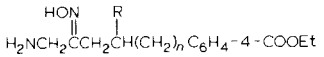

(IV.137) n = O, R = H
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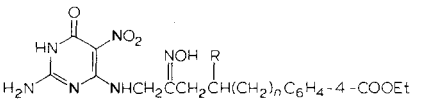
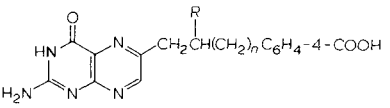
(IV.139) n = O, R = Me
(IV.140) n = O, R = Et
(IV.141) n = 1, R = Me



(IV.142) n = O, R = H
(IV.143) n = 1, R = H
(IV.144) n = O, R = Me
(IV.145) n = O, R = Et
(IV.146) n = 1, R = Me



(IV.147) n = O, R = H
(IV.148) n = 1, R = H
(IV.149) n = O, R = Me
(IV.150) n = O, R = Et
(IV.151) n = 1, R = Me

Biological activity

The ability of 10-deazafolic acid (IV.84) to inhibit DHFR was assayed against pigeon liver enzyme and compared with that of MTX [117]. The IC_{50} value of (IV.84) was found to be 6.1 μM, whereas that of MTX was 0.014 μM. 9,10-Dehydro-10-deazafolic acid (IV.90) was even less inhibitory than (IV.84), with an IC_{50} value of 85 μM. In light of what is now known about 2-amino-4(3*H*)-oxo antifolates, it would perhaps have been of interest to determine the IC_{50} values of (IV.84) and (IV.90) against other folate-requiring enzymes, especially those concerned with purine biosynthesis. Further study of these compounds was discouraged, however, by their lack of antibacterial activity against *S. faecium* and their failure to inhibit L1210 leukaemia in mice. It may be noted, however, that while (IV.84) was stated to lack activity against L1210 leukaemia, it was in fact toxic to mammalian cells, since the mice died when the dose reached 80 mg/kg on the qd × 9 schedule. It is therefore conceivable that a therapeutic effect might have been obtained if a different schedule, or other tumours, had been used.

In preliminary antibacterial assays [126], 10-methyl-10-deazafolic acid (IV.149) and 10-ethyl-10-deazafolic acid (IV.150) had IC_{50} values against *S. faecium* of 0.02 and 3.6 μM, respectively. Against *L. casei*, the activity of (IV.149) was dramatically decreased ($IC_{50} > 1$ μM) whereas the activity of (IV.150) showed a slight increase ($IC_{50} = 1.3$ μM). Biochemical and pharmacological studies on these compounds, as well as their reduced derivative, the corresponding 11-deazahomofolate analogues, and the 11-deazahomoAMT derivatives, in mammalian systems will be of considerable interest.

The enzyme inhibitory activity of 10-deazaAMT (IV.91) was evaluated initially against purified DHFR and TS from *L. casei* [118]. An IC_{50} value of 4.5 nM was obtained against DHFR, as compared with 3.3 nM for MTX. As with MTX, inhibitory activity against TS was very low ($IC_{50} > 100\ \mu M$). Of interest was that the 7,8-dihydro and 5,6,7,8-tetrahydro derivatives of (IV.91), which may be viewed as examples of dual modification of regions B and C (*vide infra*), showed moderate levels of TS inhibition, with IC_{50} values of 10 and 5.7 μM, respectively.

Assays of the antibacterial activity of (IV.91) against MTX-sensitive and MTX-resistant strains of *S. faecium* and *L. casei* were also performed [118]. The IC_{50} values against MTX-sensitive *S. faecium* and *L. casei* were 0.5 and 0.05 nM, respectively. The IC_{50} values of MTX were 0.38 nM against *S. faecium* and 0.025 nM against *L. casei*, while the corresponding values for AMT were 2.5 and 0.075 nM. It thus appears that replacement of NH by CH_2 in the bridge enhanced activity against *S. faecium* 5-fold. However, with *L. casei*, against which AMT is already very active, changing NH to CH_2 produced only a small increase in activity.

Against a partially purified DHFR preparation from pigeon liver [113], (IV.91) was twice as potent as MTX. Against H.Ep.-2 cells in culture, on the other hand, MTX was the more potent inhibitor, with an IC_{50} value of 2.4 nM as compared with 9 nM for the 10-deaza analogue. These results suggested that the cellular accumulation of (IV.91), at least in H.Ep.-2 cells may be less efficient than that of MTX.

The promising *in vitro* antitumour activity of 10-deazaAMT (IV.91) led to extensive preclinical pharmacologic studies in mice as a prelude to possible clinical trial [127]. The single-dose LD_{50} value was found to be 236 mg/kg, as compared with 28 mg/kg for AMT and 386 mg/kg for MTX. In terms of acute toxicity, therefore, (IV.91) was closer to MTX than to AMT, despite the absence of an alkyl substituent at position 10. On the q2d × 5 schedule, the LD_{10} value of (IV.91) was 15 mg/kg, whereas that of AMT and MTX was 0.4 and 18 mg/kg, respectively. The plasma clearance of orally or subcutaneously administered (IV.91) (12–48 mg/kg) was kinetically similar to that of MTX, with a 100-fold decline in plasma concentration over 24 h. However, higher peak levels of (IV.91) than of MTX were observed after oral administration of an equal dose, suggesting that the 10-deaza compound might have better bioavailability than MTX when given by mouth. Greater retention of (IV.91) in S180 sarcoma and L1210 leukaemia cells than in normal intestinal epithelial cells was demonstrated, in a pattern reminiscent of other classical antifolates [128]. The particularly good retention of (IV.91) in S180 cells was consistent with the excellent therapeutic effect of the compound against this

tumour in comparison with MTX. Kinetic analysis of the transport of (IV.91) and MTX was performed in several cell lines. The K_m for transport of (IV.91) in L1210 leukaemia, S180 sarcoma and Ehrlich sarcoma cells was uniformly 1.0–1.1 μM, while for MTX the K_m ranged from 3.8 μM (L1210 cells) to 11 μM (S180 cells). The greater efficacy of (IV.91) against the S180 tumour *in vivo* was therefore related to its improved cellular accumulation in comparison with MTX. It was also found that, while the K_m for the transport of (IV.91) by normal proliferative cells of the mouse small intestine was 90 μM, the K_m for the transport of MTX was 270 μM. Thus the K_m(intestine)/K_m(S180) ratio was roughly 4-fold greater for (IV.91) than for MTX and correlated well with the greater therapeutic index of the 10-deaza compound.

The results of a Phase I clinical trial which was carried out with (IV.91) at Memorial Sloan-Kettering Cancer Center have been reported [129]. Maximum tolerated i.v. doses were 7 mg/m^2 per day given daily for 5 doses, 15 mg/m^2 per day given twice weekly for 4–6 doses, and 3 mg/m^2 per day given by continuous infusion for 5–6 days. Mucositis was dose limiting, and mild leukopenia, thrombocytopenia, and skin rash were occasionally seen. Marked leukaemia cell kill was observed in several patients with acute leukaemia and chronic myelogenous leukaemia in blast phase. A minor response was seen in one patient with carcinoma of the gall bladder. There were indications that mucositis and myelosuppression might be less severe than with MTX, though the need for a randomized trial to address this point was noted.

While chirality at position 10 posed no problem with the 10,10-dimethyl analogue (IV.109), bioassays of the 10-alkyl derivatives (IV.107) and (IV.108) were carried out first with diastereomeric mixtures [123]. The range of IC_{50} values for inhibition of MTX-sensitive *S. faecium* and *L. casei* by (IV.91) and (IV.107)–(IV.109) was 0.2–0.4 and 0.01–0.08 μM, as compared with 0.05 and 0.01 μM for MTX. Activity against MTX-resistant strains was negligible. The IC_{50} values for inhibition of purified DHFR from *L. casei* were 20–30 nM as compared with 10 nM for MTX, while IC_{50} values for inhibition of purified TS from the same organism were, as expected, higher by four orders of magnitude. Inhibition of DHFR from L1210 cells was similar to that of AMT and MTX, with K_i values of 2–4 nM. However, the K_m for transport into L1210 cells was 0.9–1.0 μM, as compared with 1.2 μM for AMT and 3.8 μM for MTX. Maximum velocities (V_{max}) for transport were equal to, or slightly greater, than those of AMT or MTX, and there was essentially no difference in the first-order rate constant for efflux. Overall, therefore, it appeared that while the binding of the 10-alkyl-10-deaza compounds to isolated DHFR from murine cells was somewhat less tight than the binding of AMT and MTX, the ability of these compounds to cross the cell membrane might be greater. The

importance of the latter property was generally supported by the *in vitro* growth inhibition data. The IC_{50} value of of (IV.91) against L1210 cells was 0.6 μM, identical to that of AMT. The IC_{50} values for the 10-alkyl analogues (IV.107)–(IV.109) were 0.5–0.6 μM, while MTX had an IC_{50} value of 3.4 μM. Impressive antitumour activity in L1210 leukaemic mice was shown by all four 10-deaza compounds. Treatment with 3 mg/kg daily for 10 days led to ILS values ranging from 162% (IV.107) to 181% (IV.91). The 10,10-dimethyl analogue (IV.109) was less potent, giving a 143% ILS at 48 mg/kg. When the drugs were administered on a q2d × 5 schedule, 18 mg/kg of (IV.91) (the optimal dose) gave a 235% ILS, while 72 mg/kg of (IV.109) gave only a 135% ILS. A second alkyl group at position 10 was therefore clearly detrimental in the *in vivo* L1210 system.

Additional biochemical and pharmacological data were reported for the 10-deaza compounds (IV.91) and (IV.107)–(IV.109) by Sirotnak *et al* [130]. All the compounds were competitive inhibitors of [^{3}H]MTX uptake in L1210, Ehrlich, and S180 cells, with K_i values of 0.8–1.0 μM, indicating that a common transport pathway is shared with MTX and AMT. All four compounds were also approximately equipotent as inhibitors of the growth of L1210, Ehrlich and S180 cells in culture, with IC_{50} values ranging from 0.6 to 1.1 μM. The K_m values for influx into murine tumour lines versus murine intestinal epithelium were determined and expressed as the K_m(epithelial)/K_m(tumour) ratio. While this ratio for MTX and (IV.91) was < 100, it was 250–290 for (IV.107), 395–470 for (IV.108), and 420–470 for (IV.109). It was evident that, while differences in influx rate among the 10-deaza compounds were relatively small for the tumour, they were substantial for the small intestine, which in the mouse is the organ most vulnerable to the toxic action of classical antifolates. The high K_m(epithelial)/K_m(tumour) ratio of the 10-alkyl-10-deaza compounds predicted that they would be well tolerated by tumour-bearing mice.

Analysis of the plasma clearance kinetics of (IV.91) and (IV.107)–(IV.109) in mice [130] revealed an overall similarity to MTX, except that the 10,10-dimethyl analogue (IV.109) was subject to more rapid biliary and renal excretion. The rapid clearance of this compound was consistent with its lower *in vivo* potency. The pharmacokinetics of retention of the 10-alkyl-10-deaza compound (IV.108) in mouse small intestine and tumour were exactly the same as those of the parent compound (IV.91), showing that introduction of an ethyl group at position 10 does not decrease selective accumulation in the tumour.

Not surprisingly, the greater retention of (IV.108) in tumour cells than in cells of the small intestine in mice was associated with efficient formation of

non-effluxing polyglutamates [130]. In an experiment in which the extent of polyglutamylation was assayed 4 h after injection of a therapeutic dose of drug, 65% and 43% of (IV.108) was recovered as polyglutamates (mainly the heptaglutamate) from L1210 cells and S180 cells, respectively, while only 9% was recovered as polyglutamates (mainly the triglutamate) from cells of the small intestine. Likewise, the recovery of polyglutamates of (IV.91) was 40% from L1210 cells, 11% from S180 cells and 3% from small intestine. In contrast, AMT polyglutamates accounted for 79% of the recovered drug in L1210 cells, 35% in S180 cells, but a very high 28% in small intestine. The high therapeutic index of (IV.91) and (IV.108) in mice was therefore in large measure a reflection of the low capacity of the normal host tissue to convert these compounds to non-effluxing polyglutamates.

In an interesting follow-up to these polyglutamylation studies, Samuels *et al.* [131] conducted a detailed analysis of the polyglutamylation of AMT, MTX, (IV.91) and (IV.108) in two murine cell lines (L1210, S180), and a human B-cell lymphoid line (Manca) under conditions chosen to produce similar rates of drug entry. In all three cells, the rate of net intracellular polyglutamylation, irrespective of the number of glutamates added, followed the order AMT > (IV.108) > MTX. Polyglutamylation was consistently faster in the human cells, though in some instances the differences were small. When accumulation of total polyglutamates was examined as a function of time, 85–95% conversion of all four drugs to polyglutamates occurred in just 6 h in Manca cells, whereas comparable conversion in L1210 cells required 24 h. With S180 cells, on the other hand, the time needed to achieve 85–95% polyglutamylation varied depending on the drug; this cell line converted 85–95% of AMT to polyglutamates in 24 h, whereas conversion of MTX, (IV.91), and (IV.108) to polyglutamates occurred to the extent of only 50–75%. Species differences were observed also among these compounds with regard to the number of glutamates added. For example, with (IV.91) the vastly dominant polyglutamylated products in Manca cells at 24 h were those containing a total of 4 and 5 glutamyl residues (i.e., (IV.91) + G_{3-4}), whereas with (IV.108) in the same cells (IV.108) + G_2 was present in approximately the same amount as (IV.108) + G_{3-4}. This was in marked contrast to AMT, whose principal metabolite in Manca cells proved to be AMT + G_1. Different results were obtained in L1210 cells, in which (IV.91) + G_{3-5} and (IV.108) + G_{3-5} were both predominant at 24 h. It thus appeared that while replacement of NH by CH_2 and CHEt generally favoured polyglutamylation, the magnitude of this effect was somewhat cell-dependent. This suggested that the substrate specificity of folylpolyglutamate synthetase might vary from one cell line to another, and that these differences might be therapeutically exploitable if they exist between a tumour and the dose-limiting tissues of the host.

Attention was called [131] to a potentially very important consequence of the differential polyglutamylation of antifolates in various cells, namely that depending on the relative rates of formation of individual polyglutamylated species from a given drug the potency of the antifolate may vary with duration of exposure. Thus, while (IV.91) and AMT (IC_{50} = 0.56 and 0.61 μM, respectively) were essentially equipotent as inhibitors of L1210 cell growth during 72 h of continuous treatment (growth inhibition assay), (IV.91) (IC_{50} = 43 nM) was 3- to 4-fold more potent than AMT (IC_{50} = 13 nM) when the cells were exposed for only 3 h and allowed to grow out in drug-free medium (clonogenic assay). Similarly, when the effects of (IV.91) and AMT were compared in S180 cells by clonogenic assay, the latter drug proved to be 10-times more potent, whereas in the growth inhibition assay the two compounds showed no difference. It is worth noting, on the other hand, that the clonogenic assay does not necessarily yield greater 'power of resolution'; for example, while (IV.91) (IC_{50} = 1.4 nM) was 50% less potent than (IV.108) (IC_{50} = 0.89 nM) in the growth assay, the two compounds (IC_{50} = 17 and 14 nM, respectively) showed similar activity in the clonogenic assay. Thus, while it was consistently observed that the extracellular antifolate concentration needed to achieve a given level of cell kill increased as the time of exposure was decreased, the quantitative differences in potency among the members of the series were amplified in some cell lines but not in others.

Because of the need to show therapeutic activity against a broad panel of tumours whenever a new anticancer drug is under consideration for clinical trial, the 10-deaza analogues (IV.91) and (IV.107)–(IV.109) were tested *in vivo* against a number of murine tumour models [132]. In addition to L1210 leukaemia, these models included i.p. implanted S180 (ascites), C1498, Ehrlich, and Tapper (ascites) tumours, intradermally implanted S180 (solid) and Tapper (solid) tumours, and subcutaneously implanted E0771 mammary carcinoma, Lewis lung carcinoma, and T241 sarcoma. All drugs were given subcutaneously, either qd × 5 or q2d × 5. The 10-ethyl-10-deaza analogue (IV.108) was more efficacious than MTX against the L1210, S180, and C1498 tumours. At doses of 15–25 mg/kg (q2d × 5), the ILS was > 235% against L1210 (with 2 out of 20 mice surviving 60 days), > 242% against S180 (with 6 out of 20 mice surviving 60 days), and 131% against C1498. The calculated cell kill was 3.9 $\log_{10}$ units in the L1210 assay and 5.7 $\log_{10}$ units in the S180 assay. This was in marked contrast to MTX, which gave a < 1-$\log_{10}$ cell kill. The activity of the 10-methyl analogue (IV.107) was very similar to that of (IV.108), whereas that of (IV.91) was somewhat lower though still better than that of MTX. Against the S180 (solid) tumour, qd × 5 treatment with (IV.91) or (IV.108) at the LD_{10} value of 3 mg/kg produced similar 60% reductions in

tumour growth (expressed as tumour volume) relative to controls, while against the Tapper (solid) tumour there was a 96% reduction in tumour growth with both drugs. Against the subcutaneously implanted E0771 breast carcinoma, treatment at the LD_{10} of 6 mg/kg (qd × 5) produced a > 99% reduction in tumour growth when therapy was started on day 7 and a 91% reduction in tumour volume when therapy was started on day 14. The ILS for animals who eventually succumbed from metastatic disease was 102%, whereas with the same regimen of MTX there was no significant ILS at the highest tolerated dose. The 10-ethyl analogue (IV.107) was also much superior to (IV.91) in this model. However, in two solid tumours, Lewis lung carcinoma and T241 sarcoma, that were relatively insensitive to MTX, neither (IV.91) nor (IV.108) showed much activity. Other murine tumours against which (IV.108) has been found by Schmid *et al.* [133] to be very active are the metastatic EO771 mammary adenocarcinoma (83% ILS at 140 mg/kg, q4d × 3) and the metastatic T241 fibrosarcoma (64% ILS at the same dose). These tumours are minimally responsive to MTX.

In an extension of these studies from murine to human tumour models, a side by side comparison of (IV.91), (IV.108) and MTX was made against human xenografts of MX-1 mammary carcinoma, LX-1 lung carcinoma, and CX-1 colon carcinoma in athymic mice [133]. While MTX showed no activity and (IV.91) showed minimal activity in these systems, (IV.108) yielded impressive antitumour effects. Against the MX-1 tumour, qd × 5 treatment with 4.5 mg/kg of (IV.108) produced a 76% reduction in mean tumour volume 5 days after cessation of therapy, with 4 of 9 animals showing no palpable tumour. Against the LX-1 and CX-1 tumours, the same treatment with (IV.108) led to reductions in tumour volume of 47% and 30%, respectively. These results provided additional evidence of the superiority of (IV.108) over (IV.91) as a candidate for clinical use.

10-Ethyl-10-deazaAMT (IV.108) has also been found [133] to be superior to MTX in combination chemotherapy regimens against the EO771 murine mammary adenocarcinoma and T241 murine fibrosarcoma. Drugs studied included 5-fluorouracil, cyclophosphamide, cisplatin and melphalan. As is customary in such regimens, a decrease in dosage of each drug was necessary to avoid toxicity. The most effective combination was that of (IV.108) and cyclophosphamide, which gave a considerable number of long-term survivors at half the LD_{10} value of each drug alone and was superior to the combination of MTX and cyclophosphamide at the same reduction in dosage.

The toxicology, pharmacokinetics and metabolism of (IV.108) in rats and dogs have been studied very thoroughly by the Memorial Sloan-Kettering group [134]. The LD_{10} value in rats for a 3-week course by i.p. administration

was 125 mg/kg per week, while the same course in dogs, given i.v. in this case, gave an LD_{10} of only 2.5 mg/kg per week. Thus, (IV.108) was significantly more toxic to dogs than to rats. Major histopathologic findings in both species were damage to the gastrointestinal mucosa, along with some marrow hypocellularity. After a 50 mg/kg i.v. bolus in the rat, the plasma concentration of (IV.108) declined triexponentially, with half-lives for the α,β and γ phases of 6.3 min, 0.8 h and 18.5 h, respectively, and a mean residence time (MRT) of 0.7 h. Triexponential plasma pharmacokinetics were similarly observed in the dog, but the MRT appeared to be longer, ranging from 1.7 to 3.1 h in different mongrel animals. The volume of distribution was about twice the volume of total body water in rats and 2- to 3-times the volume of total body water in dogs. These values were substantially greater than has been reported for MTX. Elimination of (IV.108) in the rat was 85% complete in 48 h, with over half of the drug being excreted in the faeces. Non-renal elimination was predominant in dogs as well. By contrast, MTX is eliminated mainly in the urine. Identified metabolites of (IV.108) included the 7-hydroxy derivative and the product of cleavage of the glutamate moiety. The latter compound was presumably formed by bacterial degradation in the lumen of the gut. Tissue distribution analysis revealed that the highest concentrations of drug were in the liver, kidney, and small intestine after 0.5 and 4 h, and in the liver, kidney and colon after 24 h. Marrow and brain levels were low at all three times. Polyglutamates of (IV.108) containing one to three extra glutamates were observed in the liver, kidney, and small intestine as early as 0.5 h after drug administration. However, in contrast to the polyglutamates formed after treatment with MTX, these were not the predominant species at any time up to 72 h. On the basis of these animal studies, the dose of (IV.108) recommended for Phase I human trial was 5 mg/m^2 per week for 3 weeks, i.e., one-tenth the LD_{10} value in the dog.

The role of stereochemistry at position 10 as a determinant of biological activity in (IV.107) and (IV.108) has been rigorously documented [135]. Interestingly, the difference between stereoisomers about position 10 was found to depend on the nature of the 10-substituent, the oxidation state of ring B, and the type of bioassay. Overall, a very complex picture emerged from these studies. In assays comparing the ability of diastereomeric pairs to inhibit DHFR from L1210 cells, *l*,L-(IV.107) (K_i = 3.2 pM) was 25% more potent than *d*,L-(IV.107)(K_i = 4.2 pM). In contrast, *l*,L-(IV.108) (K_i = 12.4 pM) proved 3-times less potent than *d*,L-(IV.108) (K_i = 3.9 pM). The *l*,L-diastereomers of (IV.107) and (IV.108) (IC_{50} = 1.2 and 1.5 nM, respectively) were both less toxic than the *d*,L-diastereomers (IC_{50} = 0.7 and 0.4 nM) toward intact L1210 cells, suggesting a better correlation between toxicity and transport than

between toxicity and DHFR binding. In accord with this, the K_i for inhibition of [^{3}H]MTX transport, an indirect measure of the affinity of these drugs for the MTX/reduced folate carrier system, was lower for *l*,L and *d*,L-(IV.107) (both 1.6 μM) than for *l*,L- and *d*,L-(IV.106) (both 1.9 μM), and the ratio of K_i values for MTX transport inhibition was about the same as the ratio of IC_{50} values for cell growth inhibition. Binding to the carrier protein therefore correlated well with toxicity, and seemed to be more sensitive to the size of the hydrophobic 10-alkyl substituent than to the stereochemical configuration about position 10.

Compounds *l*,L-(IV.108) and *d*,L-(IV.108) were tested also as inhibitors of DHFR from *L. casei* and chicken liver in comparison with *rac*,L-(IV.108) [135]. The *d*,L-diastereomer was 2- to 3-fold more potent than the *l*,L-diastereomer, in qualitative agreement with the activity observed against L1210 enzyme. The racemate, as expected, showed intermediate activity. On the other hand, in antibacterial assays using *S. faecium* and *L. casei*, the *l*,L-diastereomer was the more potent one against both organisms, with the difference being greater against *S. faecium*. The superior ability of *S. faecium* to reveal small structure–activity differences within a series of compounds has been noted earlier.

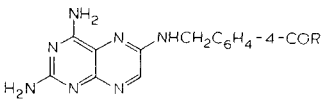
The ability of 7,8-dihydro and 5,6,7,8-tetrahydro derivatives of *l*,L- and *d*,L-(IV.107) to inhibit DHFR was also assayed [135]. Activity was increased in both the di- and tetrahydro series, and, as expected, the activity of reduced *rac*,L-(IV.108) derivatives was intermediate between that of reduced *l*,L- and reduced *d*,L-derivatives. The most potent member of the series, 7,8-dihydro-*d*,L-(IV.107), had an IC_{50} value of 25 nM against purified *L. casei* and chicken liver DHFR (versus 10 and 20 nM, respectively, for MTX) and IC_{50} values of 0.057 and 2.3 nM against *S. faecium* and *L. casei* (versus 0.028 and 0.84 nM, respectively, for MTX).

In vivo against L1210 leukaemia in mice [125], *l*,L-(IV.108) was found to produce a 187% ILS at a dose of 24 mg/kg (q2d × 5), whereas *d*,L-(IV.108) gave a 199% ILS at a dose of 12 mg/kg. Toxic deaths were seen at 32 mg/kg of *l*,L-(IV.108) as compared with 18 mg/kg of *d*,L-(IV.108). The optimal dose of MTX on the q2d × 5 schedule, 12 mg/kg, afforded only a 147% ILS. Thus, the *d*,L-diastereomer of (IV.108) was more toxic *in vivo*, just as it was *in vitro*, and was comparable to, or slightly better than, MTX in terms of therapeutic effect. Overall, however, the difference in activity between *d*,L- and *l*,L-(IV.107) was rather minor.

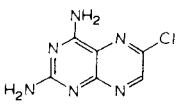
C^9–N^{10} interchange

A representative of this class of analogues was synthesized in 1974 by Nair *et al.* [136] in the form of 'isoaminopterin' (isoAMT, (IV.152)). The key intermediate in the synthesis was 2,4-diamino-6-[*N*-(4-carboxybenzyl)amino]pteridine (IV.153). Mixed anhydride coupling of (IV.153) and α-benzyl L-glutamate linked at the γ-position to Merrifield resin, followed by alkaline hydrolysis, afforded (IV.152) along with 2–3% of the corresponding 2-amino-4-oxo derivative ('isofolic acid'). The UV absorption spectrum of (IV.152) [λ_{max} (pH 13) 248, 269, 413 nm; λ_{max} (pH 1) 260, 402 nm] was, as expected, different from that of AMT. An interesting feature was the presence of peaks above 400 nm (compare the data reported for the product formulated as (IV.51); *vide supra*).

Compound (IV.153) was prepared most satisfactorily from methyl 2-amino-5-chloropyrazine-3-carboxylate via a sequence consisting of amidation, dehydration to an amino nitrile, and cyclization to 2,4-diamino-6-chloropteridine (IV.154) with guanidine. Heating with *p*-carboxybenzylamine in dimethylsulphone containing 1,4-diazabicyclo-[2.2.2]octane as a non-nucleophilic HCl scavenger converted (IV.154) to (IV.153) in 10–15% yield. An alternative, but even less efficient, route to (IV.153) began with a condensation reaction between 2,4-diamino-6-chloro-5-nitropyrimidine and 4-[(*N*-glycylamino)methyl]benzoic acid. Reduction of the nitro group with sodium hydrosulphide proceeded well, but heating in bis(2-ethoxyethyl)ether at reflux temperature in the presence of molecular sieves afforded only a low yield of cyclized product.



(IV.152) R = NHCH(COOH)CH_2CH_2COOH
(IV.153) R = OH

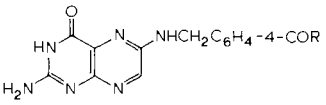


(IV.154)

Bioassays with isoAMT (IV.152) were performed against *L. casei* and *S. faecium* [136]. The IC_{50} value against *L. casei* was 9 nM as compared with 13 nM for MTX, whereas the IC_{50} value against *S. faecium* was 45 nM as compared with 60 nM for MTX. Interestingly, (IV.152) was only half as potent as MTX as an inhibitor of purified DHFR from *L. casei* even though it was more potent than MTX against the intact organism. Reversal of *L. casei* growth inhibition by (IV.152) was achieved with folic acid or thymidine, but not with methionine or adenine. This suggested that the biochemical mode of

action of isoAMT is probably at the level of thymidylate biosynthesis rather than B-12-dependent methionine biosynthesis or *de novo* purine biosynthesis.

Isofolic acid (IV.155) has likewise been synthesized and its antibacterial activity briefly described [137]. The pteridine ring system was constructed from 2-amino-4-chloro-5-nitro-6(1*H*)-oxopyrimidine and 4-[(*N*-glycylamino)methyl]benzoic acid. After reduction of the nitro group with sodium dithionite, ring closure was effected in bis(2-ethoxyethyl) ether at the reflux temperature in the presence of molecular sieves and an HCl scavenger. Oxidation of the resulting 7,8-dihydropteridine with MnO_2 yielded isopteroic acid (IV.156, 10–15%). Treatment of (IV.156) with trifluoroacetic anhydride at room temperature for several days, followed by mixed anhydride coupling to diethyl L-glutamate and alkaline hydrolysis (0.5 M NaOH, 45 min at 90 °C), afforded the desired product in 35% yield. An improved yield (75–80%) was obtained by using α-benzyl L-glutamate linked to Merrifield resin at the γ-position. Removal from the resin and hydrolysis of the benzyl ester were accomplished by treatment with 1:1 dioxan-2 M NaOH (1 h at room temperature followed by 20 min at 50 °C). The NMR spectrum of the product, taken in 0.1 M NaOD in D_2O, showed the phenyl ring protons as a pair of doublets at δ 7.48 and 7.8 and the C-7 proton as a singlet at δ 8.1. The position of the benzylic NCH_2 protons was not reported.



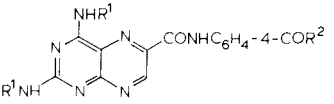
(IV.155) R = $NHCH(COOH)CH_2CH_2COOH$
(IV.156) R = OH

Antibacterial assays were carried out with isofolic acid (IV.155) against *S. faecium* and *L. casei* in the presence of 5.4 nM and 1.1 nM folic acid, respectively [137]. The IC_{50} against *S. faecium* was found to be 5.4 nM, while that against *L. casei* was 200 nM. The comparatively low activity of IV.155 against *L. casei* was unexpected, in view of the fact that this organism is generally the more sensitive one to classical antifolates of the 2,4-diaminopteridine type.

C^9 oxidation

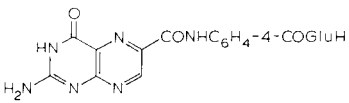
The 9-oxo derivative, (IV.157), of AMT has been synthesized [138] from 2,4-diamino-6-hydroxymethylpteridine, which on oxidation with potassium permanganate followed by treatment with trifluoroacetic anhydride yielded

the blocked and activated intermediate (IV.158). Upon reaction with *N*-(4-aminobenzoyl)-L-glutamic acid and removal of the *N*-trifluoroacetyl groups with 0.1 M NaOH (4 days at room temperature), (IV.158) was converted to (IV.157) in 30% yield. The NMR spectrum of (IV.157) in 0.1 M NaOD-D_2O showed the C-7 proton as a highly deshielded singlet at δ 9.10 (reflecting the electron-withdrawing effect of the 9-oxo substituent) and the phenyl protons as an A_2B_2 pair of doublets at δ 7.48 and 7.75. The presence of the carbonyl group in (IV.157) resulted, predictably, in a UV absorption spectrum [λ_{max} (0.1 M NaOH) 275 nm (δ 23,690), 380 (10,117); λ_{max} (0.1 M HCl) 271, 346 nm] that was different from the spectrum of AMT. Biological activity data for (IV.157) would be of interest but have not been reported.



(IV.157) R^1 = H, R^2 = NHCH(COOH)CH_2CH_2COOH
(IV.158) R^1 = $COCF_3$, R^2 = $OCOCF_3$

Similar chemistry was used by Nair and Baugh [138] to obtain 9-oxofolic acid (IV.159) in 44% yield. The NMR spectrum of IV.159 in 0.1 M NaOD-D_2O showed the C-7 proton as a highly deshielded singlet at δ 9.20 and the phenyl protons as doublets at δ 7.65 and 7.89. The UV spectrum [λ_{max} (0.1 M NaOH) 279 nm (ε 24,960), 310 (17,420), 374 (12,512)] was consistent with the assigned structure, and differed in the expected manner from that of (IV.159) in the long-wavelength region.

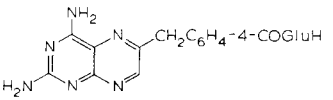


(IV.159)

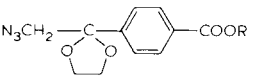
N^{10} deletion

In order to determine whether a bridge containing at least two atoms is necessary for antifolate activity, Nair *et al.* [139] prepared *N*-[4-(2,4-diamino-6-pteridinyl)methyl]benzoyl-L-glutamate ('9-nor-10-deaza-AMT' (IV.160)). 1-Bromo-3-(4-methoxycarbonylphenyl)-2-propanone was converted by a series of standard reactions to the azido ketal acid (IV.161), which was condensed with diethyl L-glutamate by the mixed anhydride method to form the diester (IV.162). Reduction of the azido group followed by reaction with 2,4-diamino-

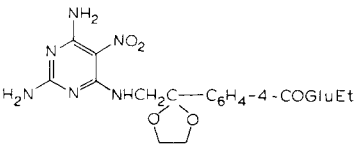
6-chloro-5-nitropyrimidine afforded the key intermediate (IV.163), which on oxidation with alkaline potassium permanganate yielded (IV.160), along with a small amount of the corresponding 2-amino-4(3*H*)-oxo analogue which could be separated by chromatography. Growth inhibition assays against *S. faecium* (IC_{50} = 5.8 nM) and *L. casei* (IC_{50} = 0.21 nM) showed (IV.160) to be a surprisingly good antibacterial agent, though less potent than MTX (IC_{50} = 0.35 and 0.035 nM, respectively) or 10-deazaAMT (IC_{50} = 0.47 and 0.047 nM, respectively). Binding to purified DHFR from *L. casei* was decreased only 3-fold in going from MTX (IC_{50} = 3.3 nM) to (IV.160) (IC_{50} = 10 nM). However, binding to DHFR from L1210 cells was substantially poorer. With the mammalian enzyme, a K_i of 2.2 nM was obtained as compared with 0.0046 nM for MTX and 0.0029 nM for 10-deazaAMT. Thus, the length of the bridge was a more important determinant of binding to the mammalian than the bacterial enzyme. Affinity for the MTX transport carrier was evaluated by performing competitive uptake experiments with [^{3}H]MTX in several murine cell lines. Whereas the K_i of unlabelled MTX in this assay ranged from 3.9 to 9.8 μM, the K_i of (IV.160) ranged from 10.9 to 13.3 μM and was consistently lower than that of MTX. These results indicated that the length of the bridge is important not only for binding to DHFR but also for efficient cell uptake, and were qualitatively consistent with the decreased ability of (IV.160) to inhibit L1210 cell growth (IC_{50} = 130 nM) in comparison with MTX (IC_{50} = 5.8 nM).



(IV.160)



(IV.161) R = OH
(IV.162) R = NHCH(COOH)CH_2CH_2COOH



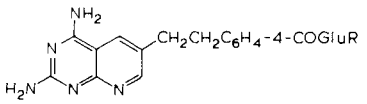
(IV.163)

COMPOUNDS MODIFIED IN THE BRIDGE AND PTERIDINE MOIETY

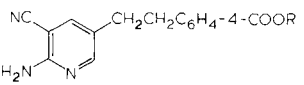
5,10-Dideaza compounds

Inspired by the very promosing preclinical and clinical activity of 10-deazaAMT and 10-alkyl-10-deazaAMT analogues, Taylor *et al.* [140] devel-

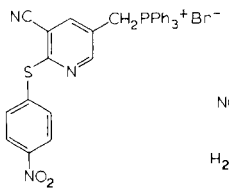
oped a synthesis of 5,10-dideazaAMT (IV.164) that made it possible to evaluate for the first time the effect of deleting nitrogen from the two positions of the molecule most intimately associated with folate metabolism. The key intermediate in the synthesis, the amino nitrile *t*-butyl ester (IV.166), was prepared in several steps from 2-(4-nitrophenylthio)-3-cyano-5-methylpyridine. Bromination of the methyl group with *N*-bromosuccinimide and subsequent reaction with triphenylphosphine gave the phosphonium bromide salt (IV.168) in 62% yield. Condensation of (IV.168) in the presence of Et_3N with the ethyl ester (88% yield) or *t*-butyl ester (81% yield) of 4-formylbenzoic acid, followed by stirring in a mixture of liquid ammonia and $CuBr_2$ under pressure at room temperature for 14 days, afforded the styrylpyridines (IV.169) (64%) and (IV.170) (84%), respectively. Upon reduction (H_2/Pd-C), (IV.170) gave (IV.166). Annulation of (IV.166) with guanidine produced the ester (IV.171) (61%), which on hydrolysis with HCOOH (79% yield) or HCl (92% yield) afforded the acid (IV.172). The same sequence from (IV.170) gave the ester (IV.174) and acid (IV.175).



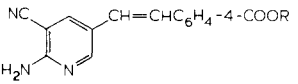
(IV.164) R = H
(IV.165) R = Et



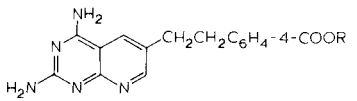
(IV.166) R = *t*-Bu
(IV.167) R = Me



(IV.168)



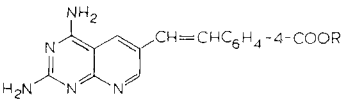
(IV.169) R = Et
(IV.170) R = *t*-Bu



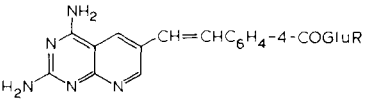
(IV.171) R = *t*-Bu
(IV.172) R = H
(IV.173) R = Me

Coupling of (IV.175) with diethyl L-glutamate or di-*t*-butyl L-glutamate in *N*-methylpyrrolidin-2-one in the presence of diphenyl chlorophosphonate and *N*-methylmorpholine led to 9,10-unsaturated diesters (IV.176) (26%) and (IV.177) (48%), respectively. Reduction of the 9,10-double bond required acid, possibly because the unprotonated pyridine nitrogen led to poisoning of the hydrogenation catalyst. Thus, (IV.177) was not reduced, though it could be cleaved satisfactorily to the styryl diacid (IV.178) with HCl in nitromethane. Catalytic hydrogenation of the diethyl ester (IV.176) in trifluoro-

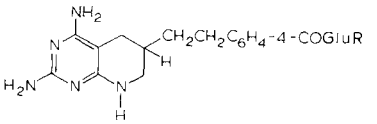
acetic acid for 24 h led to two chromatographically separable products in approximately 5:1 ratio. The major product, which was eluted more slowly from silica gel, was the diester (IV.165) (58% yield) with an intact deazapteridine ring; the faster moving minor product was formulated as the 5,6,7,8-tetrahydro derivative (IV.179). When hydrogenation was allowed to continue for 72 h, (IV.179) could be isolated in 31% yield. Alkaline hydrolysis of the ester groups in (IV.165) yielded the desired (IV.164) (44%), while hydrolysis of (IV.179) led to the tetrahydro derivative (IV.180) (42%). The NMR spectrum of (IV.164) in d_1-trifluoroacetic acid displayed singlets at δ 8.7 and 9.1 corresponding to the C-5 and C-7 protons, respectively. When the NMR spectrum of the diethyl ester (IV.165) in d_6-DMSO was examined, the C-5 and C-7 protons could not be distinguished, as they both absorbed at δ 8.6. Thus, protonation of N^8 had a deshielding influence on the proton at C-7, but not on the proton at C-5. A puzzling feature of the NMR spectrum of diethyl ester (IV.179), similarly taken in d_6-DMSO, was the presence of a singlet at δ 8.7. A possible explanation of this is that some reoxidation of (IV.179) to (IV.165) by DMSO was taking place in the NMR sample tube.



(IV.174) R = t-Bu
(IV.175) R = H

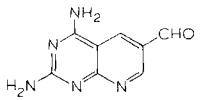


(IV.176) R = Et
(IV.177) R = t-Bu
(IV.178) R = H

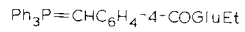


(IV.179) R = Et
(IV.180) R = H

An alternative route to the diethyl ester IV.176 was also developed, involving a slow heterogeneous reaction (room temperature, 3 weeks) between 2,4-diamino-5-deazapteridine-6-carboxaldehyde (IV.181) and the ylide (IV.182), generated from the corresponding triphenylphosphonium bromide salt with sodium hydride in *N*-methylpyrrolidone. The yield via this route was 35%.

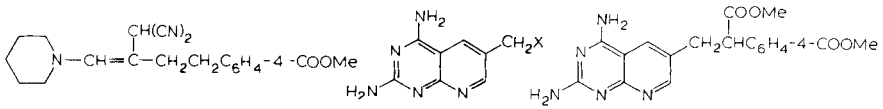


(IV.181)



(IV.182)

Another synthetic route to 5,10-dideazaAMT (IV.164), devised independently by De Graw *et al.* [140b], shares certain features with the Taylor approach [140a] but also differs in a number of respects. A key intermediate in this scheme is the amino nitrile (IV.167), which was obtained in 87% yield on treatment of the dienylidenemalonitrile (IV.183) with ethanolic ammonia at room temperature. Annulation of (IV.167) with guanidine in 2-methoxyethanol (3 days at 100 °C) resulted in concomitant hydrolysis of the ester group, giving the acid (IV.172). Mixed anhydride coupling of diethyl L-glutamate to (IV.172) afforded a low yield of the diester (IV.165), which on alkaline hydrolysis was converted to (IV.164) [λ_{max} (pH 13) 247 nm (ε 32,900), 346 (6,700)]. The key intermediate (IV.183) was obtained from 4-toluic acid in a five-step sequence consisting of (i) treatment with lithium diethylamide followed directly by alkylation with 3-methoxyallyl chloride and esterification with methyl iodide (26% yield); (ii) acid hydrolysis of the enol ether group (53% yield); (iii) formation of a piperidine enamine (90% yield); and (iv) condensation with ethoxymethylenemalononitrile (13%). Since this was a very inefficient route, an alternative scheme was also developed, in which 2,4-diamino-5-deazapteridine-6-carboxaldehyde (IV.181) was reduced with $NaCNBH_3$ to the alcohol (IV.184) (40% yield) and the latter was converted to bromide (IV.185) (94% yield) with $Ph_3P \cdot Br_2$ in DMF. The anion of dimethyl homoterephthalate, generated with sodium hydride in DMF at −40 °C, was then treated with (IV.185) to obtain the diester, (IV.186) (50% yield). Upon being heated with excess NaCN in DMSO at 180–190 °C, (IV.186) underwent ester cleavage and concomitant loss of the benzylic carboxyl group to form (IV.172) (47% yield).



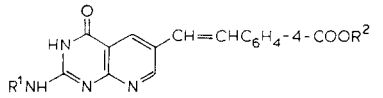
(IV.183)

(IV.184) X = OH
(IV.185) X = Br

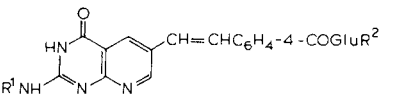
(IV.186)

For the preparation of the 2-amino-4(3*H*)-oxo analogues of this series, the 2,4-diamino-9,10-dehydro derivative (IV.175) was converted to (IV.187) (88% yield) by heating in 1 M NaOH under reflux for 3 h [140a]. Treatment of (IV.187) with acetic anhydride containing a catalytic amount of 4-(*N*,*N*-dimethylamino)pyridine afforded the 2-acetamido-4-oxo mixed anhydride (IV.188) (84%), which on careful alkaline hydrolysis was converted to the protected acid (IV.189) (77%). Coupling to diethyl L-glutamate in *N*-methylpyrrolidin-2-one in the presence of phenyl *N*-phenylphosphoramidochloridate

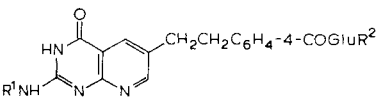
[PhNHP(=O)(OPh)Cl] and *N*-methylmorpholine gave the 9,10-dehydro derivative (IV.190) (66%). Hydrogenation of (IV.190) in trifluoroacetic acid led to a mixture of (IV.192) (56% yield) and the 5,6,7,8-tetrahydro derivative (IV.194) (18% yield), which could be separated into its components on silica gel. In contrast to the 2,4-diamino series, the tetrahydro derivative was the one eluted more slowly. Hydrolysis of (IV.190), (IV.192) and (IV.194) with methanolic NaOH (room temperature, 72 h) afforded the diacids (IV.191) (29%), (IV.193) (86%), and (IV.195) (87%), respectively. The NMR spectrum of (IV.193) in d_i-trifluoroacetic acid showed singlets at δ 8.50 and 8.90 which were assignable to the C-5 and C-7 protons, respectively; these signals were absent in the spectrum of (IV.195). In the NMR spectrum of the 2-acetamido diester (IV.190) in $CDCl_3$, the C-5 and C-7 protons gave distinct singlets at δ 8.35 and 8.77.



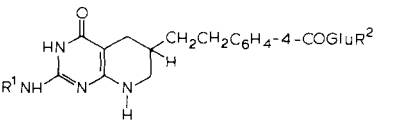
(IV.187) $R^1 = R^2 = H$
(IV.188) $R^1 = R^2 = CH_3CO$
(IV.189) $R^1 = CH_3CO$, $R^2 = H$



(IV.190) $R^1 = CH_3CO$, $R^2 = Et$
(IV.191) $R^1 = R^2 = H$



(IV.192) $R^1 = CH_3CO$, $R^2 = Et$
(IV.193) $R^1 = R^2 = H$



(IV.194) $R^1 = CH_3CO$, $R^2 = Et$
(IV.195) $R^1 = R^2 = H$

The 2,4-diamines (IV.164) and (IV.180) were evaluated as inhibitors of DHFR from beef liver and of TS from *L. casei* [140a]. The IC_{50} values of the two compounds against DHFR were 43 nM and 71 nM, respectively, while that of MTX was 1.7 nM. Thus, deletion of N^5 and replacement of the 10-NMe group by CH_2 produced a 25-fold decrease in inhibitory potency against DHFR, while ring B reduction produced an additional small decrease. As expected, neither (IV.164) (IC_{50} = 92 μM) nor (IV.180) (IC_{50} = 920 μM) was very inhibitory against TS. Somewhat surprisingly, ring B reduction decreased TS inhibition 10-fold in this series, whereas in the pteridine series the opposite is generally observed [28].

In assays of substrate activity for a partially purified folylpolyglutamate synthetase (FPGS) preparation from mouse liver, (IV.164) had an apparent

K_m of 200 ± 23 μM versus 17.6 ± 1.4 μM for AMT [140a]. Deletion of N^5 and N^{10} clearly caused a serious loss of substrate activity, though it was encouraging that this activity was still of the same order as that of folic acid itself. The apparent K_m of 47 ± 11 μM for the tetrahydro derivative (IV.180) was *ca.* 4-fold lower than that of (IV.164), indicating that reduction of ring B was favorable for FPGS catalytic activity.

Compounds (IV.164) and (IV.180) were both potent inhibitors of the growth of L1210 murine leukaemia cells in culture (continuous drug treatment), with IC_{50} values of 17 and 3.3 nM, respectively, as compared with 2.1 nM for AMT and 10 nM for MTX [140a]. The higher potency of (IV.180) relative to (IV.164) was more consistent with the FPGS substrate activity data than the DHFR inhibition data, suggesting that polyglutamylation played an important role in the cytotoxicity of these compounds. *In vivo* potency was likewise greater for (IV.180) than (IV.164). While the optimal therapeutic dose in mice with L1210 leukaemia was 4 mg/kg (dq × 9) for (IV.164), this dose for (IV.180) was only 1 mg/kg. Of interest was the fact that even though the optimal dose with MTX on the qd × 9 schedule was the same as that of (IV.164), the 5,10-dideaza analogue afforded an ILS of 130% versus only 85% for MTX.

The 2-amino-4-(3*H*)-oxo analogues (IV.193) and (IV.195), as expected, were very poor inhibitors of DHFR [140a]. Against TS, (IV.193) showed a very modest level of activity (IC_{50} = 77 μM) while (IV.195) was essentially inactive (IC_{50} > 1 mM). Both compounds, however, were superior to the corresponding 2,4-diamino derivatives as substrates for mouse liver FPGS. The apparent K_m of 9.7 ± 2.1 μM for (IV.195) was particularly impressive, since it was almost as low as that of 5,6,7,8-tetrahydrofolate, which is among the best endogenous substrates for this enzyme. This indicated that (IV.195) would be well retained in cells and might therefore have high biological activity. In agreement with this expectation the IC_{50} value against cultured L1210 cells was only 59 nM. These results were very encouraging, since they pointed to the likelihood that the 2-amino-4(3*H*)-oxo-5,6,7,8-tetrahydro derivative was killing tumour cells by inhibiting an enzyme of the folate pathway other than DHFR.

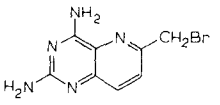
Important insights into the mode of action of (IV.195) have been gained recently through a series of biochemical and cell growth inhibition studies [141, 142]. The most significant conclusion of these studies was that this compound acts primarily as an inhibitor of an early folate-requiring enzyme in the *de novo* pathway to purines, most probably glycinamide ribotide transformylase (GAR TFase). Consistent with this view were the findings that the toxicity of (IV.195) toward cultured L1210 cells was prevented by hypoxan-

thine but not thymidine, and that incorporation of [^{3}H]deoxyuridine and [^{3}H]thymidine into DNA was unaffected at growth inhibitory concentrations of the drug. Implication of GAR TFase as the probable target enzyme was based on the observation that growth inhibition was reversed not only by hypoxanthine but also by aminoimidazole carboxamide. Impressive evidence of the broad therapeutic spectrum of (IV.195) was obtained in assays against a variety of murine solid tumours against which MTX had, at best, moderate activity. A high degree of activity (95–100% inhibition of tumour growth) was observed against X-5563 myeloma, 6C3HED lymphosarcoma and B16 melanoma, while slightly lower though still excellent activity (80–95% inhibition of tumour growth) was observed against Colon 26 carcinoma, Lewis lung carcinoma and Madison lung carcinoma [138]. Additional preclinical studies on this very interesting compound are actively ongoing in anticipation of clinical trial (Grindey, G.B., personal communication).

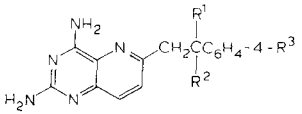
8,10-Dideaza compounds

Simultaneous deletion of N^8 and N^{10} in AMT and MTX has likewise been successfully achieved [143–145]. The key step in the synthesis of 8,10-dideaza analogues of AMT was a condensation reaction between 2,4-diamino-6-bromomethyl-8-deazapteridine (IV.196) [143, 144] and the anion of either dimethyl homoterephthalate or dimethyl α-alkylhomoterephthalates (alkyl = Me, Et, *n*-Pr) in DMF, which gave methyl 4-amino-10-methoxycarbonyl-4-deoxy-8,10-dideazapteroate (IV.197) or the corresponding 10-alkyl analogues (IV.198)–(IV.200) in yields of 52–83% depending on the size of the substituent at position 10. The success of this alkylation reaction was in sharp contrast with the inability of dimethyl homoterephthalate to alkylate 2,4-diamino-6-bromomethypteridine. Treatment of the 'extended malonate esters' (IV.197)–(IV.200) with NaCN in DMSO at 175–180 °C resulted in concomitant ester cleavage and decarboxylation to form 4-amino-4-deoxy-8-deazapteroic acid (IV.201) (96%) and the 10-alkyl derivatives (IV.202)–(IV.204). Coupling with diethyl L-glutamate in DMF via the mixed carboxylic carbonic anhydride method converted (IV.193)–(IV.197) to the diesters (IV.205) (25%), (IV.206) (54%), (IV.207) (34%), and (IV.208) (35%), respectively. Upon alkaline hydrolysis with NaOH in aqueous 2-methoxyethanol (room temperature, 3 h), the diesters yielded the diacids (IV.209)–(IV.212), the UV spectral characteristics [e.g., λ_{max} (pH 13) 238 nm (ε 37,400), 343 (95500) for (IV.210)] of which were consistent with their structure as 8,10-dideazaAMT analogues.

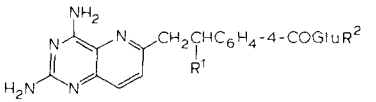
A synthesis of 8,10-dideazafolic acid (IV.213) was reported briefly by De-



(IV.196)

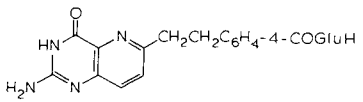


(IV.197) R^1 = R^3 = COOMe, R^2 = H
(IV.198) R^1 = R^3 = COOMe, R^2 = Me
(IV.199) R^1 = R^3 = COOMe, R^2 = Et
(IV.200) R^1 = R^3 = COOMe, R^2 = n-Pr
(IV.201) R^1 = R^2 = H, R^3 = COOH
(IV.202) R^1 = H, R^2 = Me, R^3 = COOH
(IV.203) R^1 = H, R^2 = Et, R^3 = COOH
(IV.204) R^1 = H, R^2 = n-Pr, R^3 = COOH

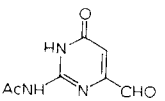


(IV.205) R^1 = H, R^2 = Et
(IV.206) R^1 = Me, R^2 = Et
(IV.207) R^1 = R^2 = Et
(IV.208) R^1 = n-Pr, R^2 = Et
(IV.209) R^1 = R^2 = H
(IV.210) R^1 = Me, R^2 = H
(IV.211) R^1 = Et, R^2 = H
(IV.212) R^1 = n-Pr, R^2 = H

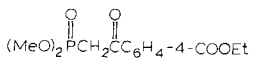
Graw *et al.* [145] in 1979. 2-Acetamido-4(3*H*)-oxopyridine-6-carboxaldehyde (IV.214) was condensed under Emmons-Horner conditions with the dimethyl β-ketophosphonate ester (IV.215), the resulting α,β-unsaturated ketone was reduced catalytically in the presence of rhodium-on-charcoal, and the ester and amide groups were removed with base to obtain the ester (IV.216). Treatment of (IV.216) with phenyldiazonium chloride gave a 5-phenylazo derivative, which on reductive cyclization in the presence of palladium-on-charcoal afforded 8,10-dideazapteroic acid (IV.217) instead of the corresponding 5,6,7,8-tetrahydro derivative, which ought to have been the product. While no reason for this unusual finding was given, it is possible that the tetrahydro compound was formed and underwent very rapid oxidation during workup (in a model experiment, both an aromatic and tetrahydro compound were obtained). Condensation of (IV.217) with diethyl L-glutamate via the mixed anhydride method followed by ester hydrolysis with warm 0.1 M NaOH yielded (IV.213).



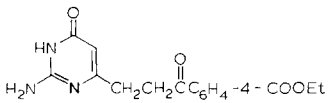
(IV.213)



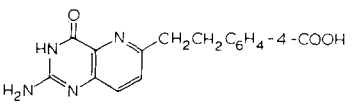
(IV.214)



(IV.215)



(IV.216)



(IV.217)

The ability of (IV.209)–(IV.212) to inhibit DHFR and TS was evaluated in comparison with MTX [144]. The IC_{50} values of all four compounds as inhibitors of DHFR from *S. faecium* and *L. casei* was in the 1–2 nM range and was essentially the same as the IC_{50} value of MTX. As expected, inhibitory potency was at least 1000-fold lower against TS than against DHFR. Antibacterial activity was evident against both organisms. Potency was greater than in the 10-alkyl-10-deazaAMT series [120] and followed the typical pattern of classical antifolates, in that *L. casei* was more sensitive than *S. faecium*. IC_{50} values of 0.09–0.54 nM were obtained against *S. faecium*, as compared with 0.3 nM for MTX, and potency appeared to vary inversely with the size of the 10-substituent (H > Me > Et > *n*-Pr). Against *L. casei*, the IC_{50} values of (IV.209)-(IV.212) were 0.007–0.04 nM as compared with 0.01 nM for MTX. Activity against MTX-resistant *S. faecium* and *L. casei* was negligible. A notable and unexpected property of the 8,10-dideaza analogues, however, was their effect on the growth of *Pediococcus cerevisiae*: whereas (IV.209)–(IV.212) gave IC_{50} values of 7–40 nM, that of MTX was 420 nM. Thus, for reasons which are not clear, deletion of nitrogen from positions 8 and 10 produced compounds as much as 60-fold more potent than MTX against this particular organism.

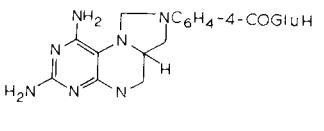
The *in vivo* biological activity of the 8,10-dideazaAMT analogues was evaluated against L1210 leukaemia in mice [144], and was found to be comparable to that of 10-deazaAMT analogues, except that potency appeared to increase with N^8 deletion. LD_{10} (q2d × 5) and ILS values ((IV.209), 0.75 mg/kg, 189%; (IV.210), 0.6 mg/kg, 203%; (IV.211), 1.5 mg/kg, 235%; (IV.212), 6.0 mg/kg, 176%) revealed increased toxicity relative to MTX (LD_{10} = 15 mg/kg) with only a modest increase in therapeutic benefit (ILS = 187%). Testing of these compounds against tumours other than L1210 leukaemia would be of interest.

Antibacterial activity has been reported for 8,10-dideazafolic acid (IV.213) [145]. In growth inhibition assays against *S. faecium* and *L. casei* at a folate concentration of 2.2 nM in the medium, IC_{50} values of 3.2 and 23 nM, respectively, were obtained. The corresponding values for MTX were 0.34 and 0.22 nM. Thus, (IV.213) was 10-fold less active than MTX against *S. faecium* but 100-fold less active against *L. casei*. Activity against tumour cells in culture was not reported.

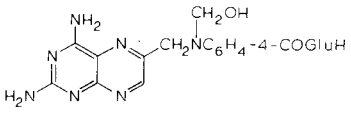
5,10-Bridged compounds

5,10-Bridged derivatives of 2,4-diaminopteridine antifolates were described in the early 1960's by Slavik *et al.* [146] in the form of 5,10-methylenetetrahydroAMT (IV.218), which was obtained from AMT by catalytic hydrogen-

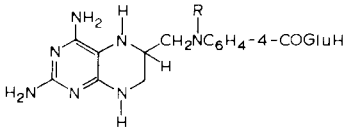
ation (H_2-Pt-AcOH-$HOCH_2CH_2OH$) followed by reaction with formaldehyde. When AMT was first converted to its 10-hydroxymethyl derivative (IV.219) and the latter was catalytically reduced, the product was not (IV.218) but a compound identified as 10-hydroxymethyltetrahydroAMT (IV.220). The 5,10-methylene and 5-hydroxymethyl derivatives could be distinguished on the basis of their UV spectra, which showed maxima at 290 and 297 nm, respectively.



(IV.218)



(IV.219)



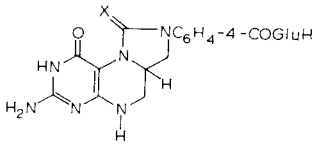
(IV.220) R = CH_2OH
(IV.221) R = H

The anti-DHFR activity of the bridged derivative (IV.218) in comparison with AMT and tetrahydroAMT (IV.221) was assayed with enzyme from pigeon liver acetone powder [147], and was also examined in liver homogenates of mice after *in vivo* drug administration [148]. The IC_{50} values of AMT, (IV.221), and (IV.218) against pigeon liver enzyme were 0.068, 5.7 and 7.8 μM, respectively [147]. In the *in vivo* assay, an AMT dose of 0.07 mmole/kg gave 50% reduction in liver DHFR activity, while the same degree of inhibition with the 5,10-methylenetetrahydro and 10-hydroxymethyltetrahydro derivatives (IV.218) and (IV.220) required doses approximately 10-fold higher [148]. Curiously, however, the LD_{50} value of (IV.218) (0.72 mg/kg) proved not to be very different from that of AMT (0.37 mg/kg) despite the substantial difference in activity between the two compounds as DHFR inhibitors. This suggested that the action of (IV.218) might involve more than just simple DHFR inhibition. Evidence in support of this view was provided [149, 150] in comparative studies of the effect of AMT and (IV.218) against AKR leukaemia in mice. The therapeutic dose range for AMT on the qd × 9 schedule against this tumour was found to be 0.05–0.3 mg/kg, whereas that of (IV.218) was 0.05–0.8 mg/kg. The therapeutic dose range for MTX was 0.2–2.3 mg/kg. At the optimal dose the 'highest average survival' increases relative to controls were: MTX, 360%; AMT, 108%; (IV.218), 62%. It thus appeared that

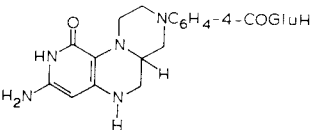
(IV.218) was a reasonably potent compound *in vivo*, considering its unimpressive activity as a DHFR inhibitor *in vitro*. Studies to determine whether this activity reflected chemical or enzymatic removal of the 5,10-methylene bridge would have been of interest.

Four interesting 5,10-bridged derivatives of folic acid with moderate activity against enzymes of the folate pathway concerned with *de novo* purine synthesis were described in 1982 by Temple *et al* [66]. For the synthesis of the first three of these derivatives, folic acid was reduced with $NaBH_4$ and the resultant product condensed directly with phosgene, thiophosgene, and cyanogen bromide to obtain 5,10-carbonyltetrahydrofolate ((IV.222), 67%), 5,10-thiocarbonyltetrahydrofolate ((IV.223), 80%), and 5,10-imidocarbonyltetrahydrofolate ((IV.224), 25%), respectively. These analogues could be viewed as being structurally related to 5,10-methenyltetrahydrofolate, since the bridge was in every case an sp_2 carbon. The UV spectra of (IV.222) [λ_{max} (0.1 M HCl) 283 nm (ε 22,900); λ_{max} (pH 7) 290 nm (ε 22,200); λ_{max} (0.1 M NaOH) 276 nm (ε 21,900)], (IV.216) [λ_{max} (0.1 M HCl) 281 nm (ε 23,400); λ_{max} (pH 7) 217 nm (ε 35,100), 285 (20,900); λ_{max} (0.1 M NaOH) 276 nm (ε 17,900), 295 sh (17,000)], and (IV.217) [λ_{max} (0.1 M HCl) 220 nm (ε 23,100, sh), 280 (23,400); λ_{max} (pH 7) 222 nm (ε 32,400), 283 (21,800); λ_{max} (0.1 M NaOH) 284 nm (ε 26,500)], were consistent with the assigned structures and were notable in providing information about possible variations in the torsional angle of the phenyl ring as a function of X in the C=X bridge. The fourth compound, a second type of bridged derivative was obtained by reductive alkylation of tetrahydrofolate with 40% aqueous glyoxal in the presence of sodium cyanoborohydride. The product (IV.225) could be viewed as a ring-enlarged homologue of 5,10-methylenetetrahydrofolate. The UV spectrum of (IV.225) [λ_{max} (0.1 M HCl) 217 nm (ε 28,900), 269 (21,600), 290 sh (19,300); λ_{max} (pH 7) 216 (ε 29,500), 287 (26,800); λ_{max} (0.1 M NaOH) 285 nm (ε 23,900)] differed from the spectra of (IV.222)–(IV.224) in a manner consistent with the fact that (a) the bridge was now an sp^3 carbon and (b) the ring generated by joining N^5 and N^{10} was six-membered rather than five-membered. In assays against enzymes of the folate pathway, the N^5,N^{10}-carbonyl derivative, (IV.222), was found to inhibit 5,10-methylenetetrahydrofolate dehydrogenase from pig liver with an IC_{50} value of *ca.* 0.1 μM and 5,10-methenyltetrahydrofolate cyclohydrolase from L1210 tumour cells with an IC_{50} of *ca.* 1 μM. Neither (IV.223) nor (IV.224) was as active as (IV.222) against these enzymes, while the N^5,N^{10}-ethano derivative (IV.225) was virtually devoid of activity. Very modest activity ($IC_{50} > 100\,\mu M$) was noted in some instances against the enzymes DHFR, TS, serine hydroxymethyl transferase, 5.10-methylenetetrahydrofolate reductase, methionine synthetase,

GAR TFase, and AICAR TFase. Because these enzyme assays suggested that (IV.222) might have the ability to interfere with purine synthesis by blocking conversion of 5,10-methylene- to 5,10-methenyltetrahydrofolate, the anti-tumour activity of this compound was evaluated *in vivo* against P388 leukaemia in mice in combination with MTX (3 mg/kg). Over a range of doses from 100 to 400 mg/kg, however, (IV.222) and MTX proved less effective in prolonging survival than MTX alone. This antagonism could have stemmed from a number of reasons including hydrolysis of the drug to leucovorin. Whatever the reasons for the antagonism between (IV.222) and MTX might have been, further work on 5,10-bridged derivatives of tetrahydrofolate as therapeutic agents did not seem worthwhile.



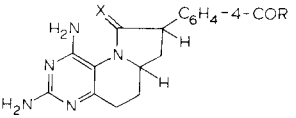
(IV.222) X = O
(IV.223) X = S
(IV.224) X = NH



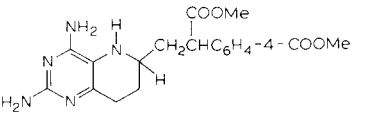
(IV.225)

The availability of IV.197 [135] (*vide supra*) has provided a convenient route to the novel analogue 5,10-methylene-5,6,7,8-tetrahydro-8,10-dideazaAMT (IV.226) [151]. This structure was viewed as a potential dual inhibitor of DHFR and TS, with the 2,4-diaminopyrimidine moiety conferring DHFR affinity, while the 5,6,7,8-tetrahydro system and 5,10-methylene bridge should favour TS binding. Catalytic hydrogenation of (IV.197) over PtO_2 in the presence of trifluoroacetic acid afforded the 5,6,7,8-tetrahydro derivative (IV.230) (83%), which on being heated at 175–180 °C without solvent for 45 min cyclized smoothly to the lactam, (IV.227) (95%). Further reaction with phosphorus pentasulphide in pyridine proceeded in low yield (10%) to the thiolactam (IV.228), but attempted dethiations with Raney nickel were mainly unsuccessful. The lactam ester (IV.227) was hydrolyzed in 87% yield to the lactam acid (IV.229) with NaOH in aqueous 2-methoxyethanol, and (IV.229) was condensed with diethyl L-glutamate in DMF by the mixed carboxylic carbonic anhydride method to form, in only 11% yield, the lactam diester (IV.231). Alkaline hydrolysis gave 5,10-carbonyl-5,6,7,8-tetrahydroAMT ((IV.232, 33%), which on successive reduction with borane in THF and alkaline hydrolysis afforded, respectively, the 5,10-methylene derivatives (IV.233) (20%) and (IV.226) (68%). Ultraviolet spectra of (IV.232) [λ_{max} (pH 13) 246 nm (ε 21,360), 297 (10,030)] and (IV.226) [λ_{max} (pH 13) 225 sh

(ε 16,700), 242 (18,800), 305 (5,100); λ_{max} (pH 1) 232 (ε 29,200), 310 (4,000)] were notable for the fact that the 5,10-carbonyl compound was the one with longer wavelength absorption at pH 13, even though it could be viewed as a 5-carbamoyl as opposed to 5-amino derivative of the 2,4-diaminopyrimidine chromophore.



(IV.226) X = H_2, R = GluH
(IV.227) X = O, R = OMe
(IV.228) X = S, R = OMe
(IV.229) X = O, R = OH
(IV.230) X = O, R = GluEt
(IV.231) X = O, R = GluH
(IV.232) X = H_2, R = GluEt



(IV.233)

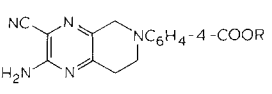
Compounds (IV.226) and (IV.231) unfortunately proved to be poor inhibitors of both DHFR (IC_{50} = 1.5–2.0 μM) and TS (IC_{50} > 20 μM) from *L. casei*, and also showed low antibacterial activity in comparison with MTX [147]. The IC_{50} value of (IV.226) as an inhibitor of L1210 cell growth in culture was 0.25 μM versus 2.5 nM for MTX. The 5,10-carbonyl derivative (IV.231) was even less active (IC_{50} > 10 μM). While these results clearly showed a one-carbon bridge between positions 5 and 10 to be detrimental to biological activity in the 2,4-diamino series, they did not invalidate the possibility that similar 2-amino-4(3*H*)-oxo compounds might still show activity.

7,10-Bridged compounds

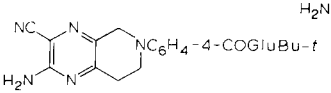
Examples of this type of structural modification were described in 1985 by Taylor *et al.* [152, 153]. The rationale for the preparation of these compounds was that metabolic oxidation at position 7 of the pyrido[3,4-*g*]pyrimidine ring system would be blocked and that the extra CH_2 groups in the 7,10-bridge would enhance lipophilicity. It was hoped that these 'tied back' analogues might show improved enzyme binding properties and increased therapeutic potency.

Condensation of the pyrrolidine enamine of *N*-[4-(*t*-butyloxycarbonylphenyl)]piperidone and (chloromethylene)malononitrile followed by reaction with ammonia in methanol at room temperature afforded the amino nitrile (IV.234) in 72% overall yield [148]. Cleavage of the *t*-butyl ester was accomplished readily with HCl in nitromethane, and the resulting acid ((IV.235), 77% yield) was coupled to di-*t*-butyl L-glutamate with the aid of diphenyl

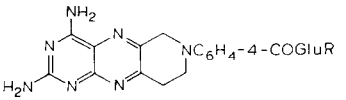
phosphorochloridate and Et_3N in hot *N*-methylpyrrolidin-2-one solution to obtain the diester (IV.236) (71%). Annulation with *N,N*-dimethylguanidine in refluxing *t*-butyl alcohol and ester hydrolysis with HCl in nitromethane then led to the diester (IV.237) (69%) and DL-7,10-ethano-5-deazaAMT ((IV.238), 88%), respectively. Although formal proof that (IV.238) was racemic about the α-carbon was not given, it is assumed to have taken place during the annulation step. Racemization of the esterified glutamate moiety in the presence of the strong base guanidine has been reported previously in the 2,4-diaminopteridine series [110], and is likely to be a general problem in this type of synthesis.



(IV.234) R = *t*-Bu
(IV.235) R = H

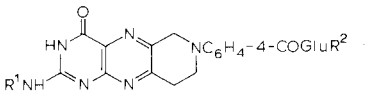


(IV.236)

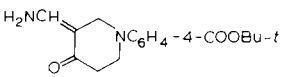


(IV.237) R = *t*-Bu
(IV.238) R = H

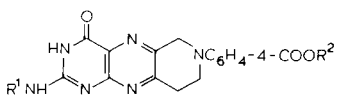
For the synthesis of the 2-amino-4-oxo (i.e., folate) analogue (IV.239), *N*-[4-(*t*-butyloxycarbonyl)phenyl]piperidone was converted to the 2-aminomethylene derivative (IV.241) (32% overall yield) by consecutive reactions with ethyl formate, dimethylsulphate, and ammonia, and (IV.241) was condensed with 2,4-diamino-6(1*H*)-pyrimidinone in refluxing acetic acid containing a drop of piperidine to obtain the *t*-butyl ester (IV.242) (51%) [147]. The 2-amino group in (IV.242) was acetylated and the ester group was cleaved with HCl in nitromethane to obtain the protected acid (IV.243) in a two-step yield of 75%. Upon reaction with diethyl L-glutamate in the presence of the coupling reagent *N*-phenylphosphoramidochloridate [$PhNHP(=O)Cl_2$], (IV.243) yielded the 2-acetamido diester (IV.240) (55%), which on hydrolysis, first with HCl in nitromethane to deprotect the 2-amino group and then with NaOH to remove the ester groups, gave L-7,10-ethano-5-deazafolic acid (IV.239).



(IV.239) $R^1 = R^2 = H$
(IV.240) $R^1 = CH_3CO$, $R^2 = H$



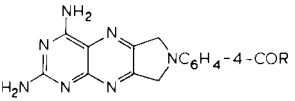
(IV.241)



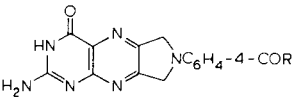
(IV.242) $R^1 = H$, $R^2 = t$-Bu
(IV.243) $R^1 = CH_3CO$, $R^2 = H$

In an extension of the work on 7,10-ethano derivatives of AMT and folate, the analogous 7,10-methano derivatives (IV.244) and (IV.246) were similarly

synthesized [153] via the 'tied back' 7,10-methano-5-azapteroic acid analogues (IV.245) and (IV.247). Although the latter compounds were successfully obtained, their extreme lack of solubility blocked further synthetic elaboration.



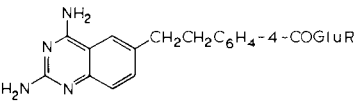
(IV.244) R = NHCH(COOH)CH_2CH_2COOH
(IV.245) R = OH



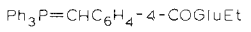
(IV.246) R = NHCH(COOH)CH_2CH_2COOH
(IV.247) R = OH

5,8,10-Trideaza compounds

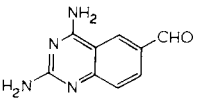
A straightforward synthesis of 5,8,10-trideazaAMT (IV.248) was accomplished [154] by a Wittig reaction between 2,4-diaminoquinazoline-6-carboxaldehyde (IV.250) and the triphenylphosphorane (IV.251). The resulting styryl derivative (IV.252) (73%) was reduced catalytically to ester (IV.249) (70%) and the latter was hydrolyzed with potassium carbonate in aqueous alcohol to form the diacid (IV.248) (78%).



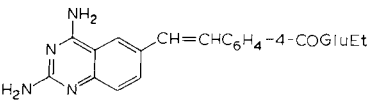
(IV.248) R = H
(IV.249) R = Et



(IV.251)



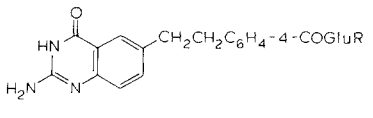
(IV.250)



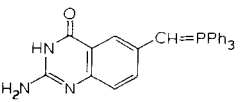
(IV.252)

Compound IV.248 was only moderately effective against L1210 leukaemia in mice, giving a 70% ILS at the optimal dose of 6.25 mg/kg (qd × 9) [154]. However, the fact that the dose producing a statistically significant effect (> 25% ILS) was as low as 0.08 mg/kg was of interest. It is possible that improved therapeutic results would have been obtained on a different treatment schedule such as q2d × 5 or q3d(days 1,4,7).

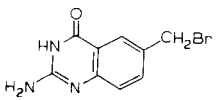
A synthesis of 5,8,10-trideazafolic acid (IV.253) was described in 1977 by Oatis and Hynes [155]. 2-Amino-6-bromomethylquinazolin-4(3*H*)-one (IV.255) was converted to the corresponding triohenylphosphorane (IV.256) and the latter was condensed with diethyl *N*-(4-formylbenzoyl)-L-glutamate in the presence of NaOEt to obtain the 6-styrylquinazoline (IV.257) as a mixture of *cis* and *trans* isomers. Reduction with H_2-Pt afforded the diester (IV.254), which on alkaline hydrolysis was converted to (IV.253). In assays of anti-tumour activity against L1210 leukaemia in mice, a single i.p. dose of 150 mg/kg of (IV.253) gave a marginal increase in lifespan of 23%. Since toxicity was not observed at 150 mg/kg, it is possible that a higher single dose or a different dose schedule might have produced better results.



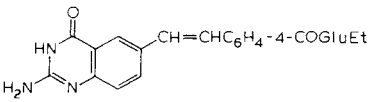
(IV.253) R = H
(IV.254) R = Et



(IV.256)



(IV.255)

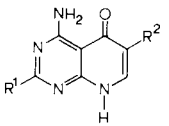


(IV.257)

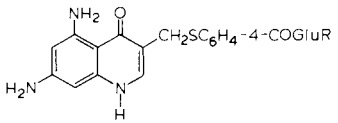
5-Deaza compounds with O or S at position 10

A novel 10-thia-5-oxo analogue of 5-deazaAMT was synthesized in 1980 by Srinivasan and Broom [156] with the aim of gaining empirical information about the nature of the steric and electronic requirements at C-5 for tight binding to DHFR. 2,4-Diamino-6-bromomethyl-5(8*H*)-oxopyrido[2,3-*d*]pyrimidine (IV.258) was prepared from ester (IV.259) in four steps consisting of (i) lithium aluminium hydride reduction, (ii) oxidation of the methylthio group to a sulphoxide/sulphone mixture followed directly by amination, and (iii) bromination with HBr in dioxan. Condensation of (IV.258) with the sodium salt of diethyl (*p*-mercaptobenzoyl)-L-glutamate gave a 58% yield of the diester (IV.260), which on hydrolysis with 1 M NaOH in aqueous ethanol (room temperature, 48 h) afforded the diacid (IV.261) in 57% yield. The UV spectrum of (IV.261) [λ_{max} (pH 1) 258 nm (ε 35,000), 281 (18,000); λ_{max} (pH 7) 256 nm (ε 31,000), 281 (17,600); λ_{max} (pH 11) 257 nm (ε 26,500), 289 (18,000)]

was consistent with the formulation of ring B as a vinylic lactam. The NMR spectrum, taken in d_6-DMSO, revealed the CH_2S protons of the bridge as a singlet at δ 4.16 and the vinylic C-7 proton as a singlet at δ 7.73. Biological activity data for (IV.261) were not reported.



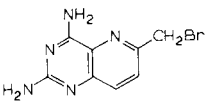
(IV.258) R^1 = NH_2, R^2 = CH_2Br
(IV.259) R^1 = MeS, R^2 = CO_2Et



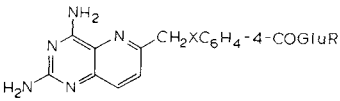
(IV.260) R = Et
(IV.261) R = H

8-Deaza compounds with O or S at position 10

10-Oxa analogues of 8-deazaAMT and 8-deazafolic acid were described in 1981 by Srinivasan and Broom [157] in an effort to determine what effect this dual modification of the classical antifolate structure might have on biological activity. Condensation of 2,4-diamino-6-bromomethylpyrido[3,2-*d*]pyrimidine (IV.262) with diethyl *N*-(4-hydroxybenzoyl)-L-glutamate in the presence of sodium ethoxide followed by hydrolysis with NaOH afforded a 52% yield of the diester (IV.263) (52%) and diacid (IV.264) (62%), respectively. The NMR spectrum of (IV.264) in d_6-DMSO-D_2O, showed the 9-CH_2O protons as a singlet at δ 5.43, the phenyl protons as a pair of doublets at δ 7.31 and 8.05, and the C-7 and C-8 protons as a single unresolved peak at δ 7.93. The UV spectral data for (IV.264) [λ_{max} (pH 13) 250 nm (ε 36,000), 343 (6,300); λ_{max} (pH 7) 248 nm (ε 35,500), 333 (6,600); λ_{max} (pH 1) 246 (ε 32,000), 318 (8,300)] were consistent with the 2,4-diaminopyridol[3,2-*d*]pyrimidine structure.



(IV.262)



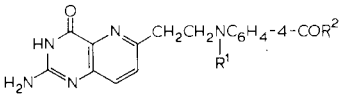
(IV.263) X = O, R = Et
(IV.264) X = O, R = H
(IV.265) X = S, R = Et
(IV.266) X = S, R = H

An analogous scheme starting from (IV.262) and the sodium salt of diethyl *N*-(4-mercaptobenzoyl)-L-glutamate [157] led to the diester (IV.265) (55%) and diacid ((IV.266), 68%). The NMR spectrum of (IV.266) in d_6-DMSO-D_2O, showed the CH_2S protons as a singlet at δ 4.63, the phenyl

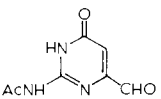
protons as a pair of doublets at δ 7.66 and 8.03, and the C-7 and C-8 protons as a resolved pair of doublets at δ 7.98 and 8.01. The ability of the C-7 and C-8 protons to be detected individually in the NMR spectrum of (IV.266) but not (IV.264) was noteworthy in that it suggested long-range interaction between the hetero atom at position 10 and the proton at position 7. The UV spectra of (IV.266) [λ_{max} (pH 13) 277 nm (ε 20,000), 343 (5,500); λ_{max} (pH 7) 277 nm (ε 16,000), 335 (5,500); λ_{max} (pH 1) 243 (ε 21,900), 275 (16,000), 320 (6,600)] were consistent with the bathochromic effect of sulphur on the *N*-benzoyl chromophore. Biological and biochemical studies with compounds (IV.264) and (IV.266) have not yet been reported.

8-Deaza compounds with carbon insertion in the bridge

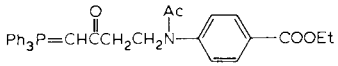
DeGraw *et al.* [158] have produced an interesting example of this type of structure modification in the form of 8-deazahomofolic acid (IV.267). 3-[*N*-Acetyl-*N*-(4-ethoxycarbonylphenyl)amino]propionyl chloride was treated successively with diazomethane, hydrogen chloride, triphenylphosphine, and sodium carbonate to obtain the stabilized ylide (IV.269). Condensation of (IV.269) with silylated 2-acetamido-4(3*H*)-oxopyrimidine-6-carboxaldehyde (IV.270) followed by catalytic hydrogenation in the presence of rhodium-on-charcoal then gave the ketone (IV.271), which on selective acid hydrolysis (HCl-EtOH) of the 2-acetamido group followed by reaction with phenyldiazonium chloride was converted to the 5-phenylazo derivative (IV.272). Hydrogenation of (IV.272) in the presence of Pd-C yielded 11-acetyl-8-deazahomopteroic acid (IV.268), apparently via oxidation during the workup. Condensation of (IV.268) with diethyl L-glutamate by the mixed anhydride method, followed by alkaline deprotection, afforded (IV.267).



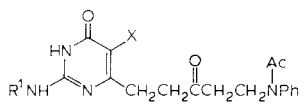
(IV.267) R^1 = H, R^2 = NHCH(COOH)CH_2CH_2COOH
(IV.268) R^1 = Ac, R^2 = H



(IV.270)



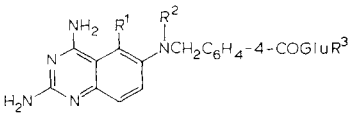
(IV.269)



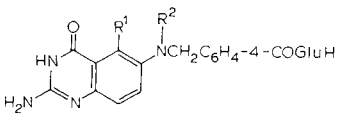
(IV.271) R^1 = Ac, X = H
(IV.272) R^1 = H, X = $N_2C_6H_5$

5,8-Dideaza compounds with C^9-N^{10} interchange

Compounds exemplifying replacement of N^5 and N^8 by carbon with concomitant interchange of C^9 and N^{10} have been prepared and studied by Hynes *et al.* [41, 43, 44, 53, 54, 57, 60]. Condensation of 2,4,6-triaminoquinazoline or 2,4,6-triamino-5-methylquinazoline with diethyl *N*-(*p*-formylbenzoyl)-L-glutamate in the presence of hydrogen and Raney nickel gave diesters which, on alkaline hydrolysis, were converted to 5,8-dideazaisoAMT (IV.273) and 5-methyl-5,8-dideazaisoAMT (IV.274), respectively [41]. The diesters could also be obtained by forming a Schiff's base between the amine and aldehyde and subsequently reducing the C=N double bond with dimethylamine borane in acetic acid. 5-Chloro-5,8-dideazaisoAMT (IV.275) was prepared in similar fashion [44]. When 2,6-diamino-, 2,6-diamino-5-methyl, and 2,6-diamino-5-chloro-4(3*H*)-quinazolinones were used, 5,8-dideazaisofolic acid (IV.281, IAHQ) and its 5-methyl and 5-chloro analogues (IV.282) and (IV.283), respectively, were obtained [41]. In a subsequent study, di-*t*-butyl *N*-(*p*-formylbenzoyl)-L-glutamate was used in place of the diethyl ester, and ester cleavage was achieved with trifluoroacetic acid instead of base [60]. Advantages of this procedure were that the coupling step yielded cleaner product and that ester hydrolysis was performed under non-racemizing conditions. Treatment of (IV.273) with HCOOH-Ac_2O at room temperature led to the 9-formyl derivative (IV.276) (69% yield), while reductive methylation of (IV.273) and (IV.274) with formaldehyde and sodium cyanoborohydride at pH 6.5 gave the 9-methyl and 5,9-dimethyl derivatives (IV.277) (95%) and (IV.278) (70%),



(IV.273) $R^1 = R^2 = R^3 = H$
(IV.274) R^1 = Me, $R^2 = R^3 = H$
(IV.275) R^1 = Cl, $R^2 = R^3 = H$
(IV.276) $R^1 = R^3 = H$, R^2 = CHO
(IV.277) $R^1 = R^3 = H$, R^2 = Me
(IV.278) $R^1 = R^2$ = Me, $R^3 = H$
(IV.279) $R^1 = R^2 = H$, R^3 = Et
(IV.280) R^1 = Me, $R^2 = H$, R^3 = Et



(IV.281) $R^1 = R^2 = H$
(IV.282) R^1 = Me, $R^2 = H$
(IV.283) R^1 = Cl, $R^2 = H$
(IV.284) $R^1 = H$, R^2 = Me
(IV.285) $R^1 = R^2$ = Me
(IV.286) $R^1 = H$, R^2 = CHO

respectively [59]. Similar reactions were used to synthesize the 9-methyl (IV.284), 5,9-dimethyl (IV.285), and 9-formyl (IV.286) derivatives of 5,8-dideazaisofolate. The NMR spectrum of (IV.274) in deuteriotrifluoroacetic acid showed the NCH_2 and α-CH protons as part of an unresolved multiplet at δ 4.9–5.2 and the seven aromatic protons as a multiplet at δ 7.5–8.4. In the NMR spectrum of (IV.274), the aromatic CH_3 and glutamate CH_2CH_2 gave overlapping signals at δ 2.3–3.0. The 9-formyl and 9-methyl protons in (IV.276) and (IV.277) gave prominent singlets at δ 8.3 and 3,6, respectively.

As expected, 2,4-diaminoquinazolines (IV.273) and (IV.274) proved very active as inhibitors of partly purified DHFR from rat liver and *S. faecium*, with IC_{50} values of 26 and 5.6 nM against the former enzyme and 32 and 8.3 nM against the latter [54]. The corresponding values for MTX inhibition were approximately 1 nM. Thus C^9–N^{10} transposition in the 2,4-diaminoquinazoline series was well tolerated by the bacterial enzyme, but somewhat less by the mammalian enzyme. 5-Methyl substitution appeared to favour binding, as was the case in the 'normal' 5,8-dideazaAMT series. The corresponding diesters (IV.279) and (IV.280) likewise were tightly bound to the enzyme. In another study [43], inhibition of purified DHFR from murine leukaemia cells by (IV.273)–(IV.275) was compared with inhibition by the corresponding 5,8-dideazaAMT analogues. The 5-chloro compound (IV.275) (IC_{50} = 45 nM) showed binding comparable to MTX (IC_{50} = 45 nM) and 2,4-diamino-5-chloro-5,8-dideazaAMT (IC_{50} = 46 nM), while IV.273 (IC_{50} = 60 nM) and the 5-methyl analogue (IV.274) (IC_{50} = 63 nM) were somewhat less inhibitory. Finally, in a more recent study [47], the binding of 2,4-diamino-5,8-dideaza compounds to 'form 2' DHFR from L5178Y cells was evaluated. This DHFR isozyme is of interest because of its relative insensitivity to MTX (IC_{50} = 1000 nM). The binding of compound (IV.273) (IC_{50} = 1100 nM) was comparable to that of MTX, but the N^{10}-formyl derivative (IV.276) (IC_{50} = 540 nM), the 5-chloro derivative (IV.275) (IC_{50} = 300 nM), and especially the N^9-methyl derivative (IV.277) (IC_{50} = 60 nM) were better inhibitors. While the potency of (IV.277) against the MTX-resistant 'form 2' isozyme was still not so high as that of MTX against the sensitive 'form 1' isozyme, the results obtained with this compound gave reason to hope that some other member of the series will ultimately be found to inhibit both isozymes equally. This could be an important step toward overcoming MTX resistance.

In assays against *S. faecium* DHFR, (IV.273) and (IV.274) had IC_{50} values of 0.032 and 0.0083 μM, respectively, while the diesters (IV.279) and (IV.280) had comparable or, in the latter instance, somewhat greater activity [54]. In assays against cultured L1210 murine leukaemic cells, (IV.280) had an IC_{50}

value of 1.5 μM, as compared with 0.0012 μM for MTX, whereas (IV.274) and (IV.275) had IC_{50} values of 0.46 and 0.13 μM, respectively. Thus, C^9–N^{10} transposition resulted in decreased growth inhibition against L1210 cells, although introduction of lipophilic 5-substitution partially overcame this unfavourable effect. An interesting property of the three 5,8-dideaza compounds was that they were uniformly more active (IC_{50} = 42, 54, and 15 μM, respectively) than MTX (IC_{50} = 205 μM) against L1210/R81 cells, which owe their high MTX resistance to an extreme defect in MTX active transport, along with a 35-fold elevation in DHFR [49].

As with other classical antifolates, the ability of 5,8-dideazaisoAMT (IV.273) and its 5-substituted derivatives to cross the cell membrane is an important determinant of growth-inhibitory potency. Susten *et al* [50] addressed this issue by measuring the kinetics of transport of [^{3}H]MTX in the presence of the quinazolines. The IC_{50} of IV.273 as an inhibitor of 1.0 μM MTX influx was 1.6 μM, whereas the IC_{50} values of (IV.274), (IV.275), and MTX (unlabelled) were 2.0, 1.4, and 1.0 μM. Thus substitution at position 5 caused a slight decrease in binding of the quinazolines for the MTX/reduced folate carrier protein.

5,8-DideazaisoAMT (IV.273) was tested to as inhibitor of the growth of several human gastrointestinal adenocarcinoma cell lines (HuTu80, HT29, SW480, WIDR) in culture, and was found to have IC_{50} values of 0.15–0.20 μM as compared with 0.6–1.1 μM for 5,8-dideazaAMT and 0.009–0.025 μM for MTX [60]. Thus, $NHCH_2$ was somewhat superior to CH_2NH as a 9,10-bridge in the 2,4-diaminoquinazoline series, though activity still fell far short of MTX.

In vivo antitumour assays were performed with 9-methyl-5,8-dideazaisoAMT (IV.277) against L1210 in mice [43]. Subcutaneous injection at a dose of 20 mg/kg (q2d × 5) starting on day 1 after intraperitoneal tumour inoculation produced a 169% ILS, as compared with a 161% ILS with 10 mg/kg of MTX on the same schedule. Thus, *in vivo* activity against L1210 leukaemia appeared to be undiminished by simultaneous deletion of N^5 and N^8 and replacement of the CH_2NMe bridge by $NMeCH_2$.

The 2-amino-4(3*H*)-oxo compound IAHQ (IV.281) has been reported by Scanlon *et al.* [159] and by Fernandes *et al.* [160] to be a reasonably potent inhibitor of purified TS from L1210 cells. With (6-*R,S*)-5,10-methylenetetrahydrofolate as the substrate, IAHQ behaved as a linear non-competitive inhibitor with a K_i(app) of 8.4 μM [160]. Binding of dUMP to TS was stimulated by IAHQ and, to a greater degree, by 5,8-dideazaisopteroyl triglutamate. Scatchard plots showed that one molecule of dUMP was bound per mole of enzyme. Dissociation of dUMP from the ternary complex was found in

equilibrium dialysis experiments to have a K_D of 1.2 μM in the presence of IAHQ and 0.041 μM in the presence of 5,8-dideazaisopteroyl triglutamate. This suggested that inhibition of TS in cells was dependent on conversion of the drug to polyglutamates. However, there appeared to be a qualitative difference between the mode of interaction of 5,8-dideazaisopteroyl triglutamate with TS and that of MTX polyglutamates. Whereas the latter can bind to free TS even in the absence of dUMP, the quinazolines do not bind until dUMP has formed a binary complex with the enzyme.

Since the K_i(app) of IAHQ of 0.7 μM against purified DHFR from L1210 cells was 12-fold lower than the K_i(app) against TS, it might be thought that the primary target of the compound would be DHFR. However, the authors pointed out [160] that since DHFR activity was present in considerable excess of TS activity in L1210 cells, IAHQ would be unlikely to deplete reduced folate pools sufficiently to kill cells. Moreover, inhibition of TS should also expand the dihydrofolate pool, thereby further diminishing the significance of DHFR inhibition. From this analysis it was predicted that cell growth arrest by IAHQ should be principally due to TS rather than DHFR inhibition. This was consistent with the observation that 10 μM dThd completely protected HCT-8 cells from the toxic effects of 5 μM IAHQ.

The binding of [^{3}H]FdUMP to TS isolated from L1210 cells and purified by affinity chromatography has been studied in the presence of a saturating concentration of IAHQ (IV.281) versus a saturating concentration of (6-*R,S*)-5,10-methylenetetrahydrofolate [160]. While (6-*R,S*)-5,10-methylenetetrahydrofolate stimulated the binding of 2 mol FdUMP/mol enzyme in the ternary complex, this ratio with IAHQ as the ligand dropped to only 0.1. In order to test the possibility that ternary complex formation might be enhanced by additional glutamate residues as is true with the natural cofactor, 5,8-dideazapteroyltriglutamate was used in place of IAHQ. The amount of FdUMP bound per molecule of TS did increase, but only to 0.4. Thus, binding of IAHQ and its triglutamate appeared to be easily reversible, presumably because a methylene bridge between the quinazoline and FdUMP could not form. The inability of IAHQ to significantly stimulate FdUMP binding to TS was confirmed by showing that IAHQ and 5-fluorouracil were antagonistic when used in combination against the human colon adenocarcinoma line HCT-8. Antagonism was ascribed to the fact that formation of a ternary complex between TS, FdUMP, and endogenous 5,10-methylenetetrahydrofolate is inhibited.

Another enzyme recently shown [62] to be competitively inhibited by IAHQ is aminoimidazole-4-carboxamide ribonucleotide transferase (AICAR TFase), which catalyzes the final step in the biosynthesis of purine nucleo-

tides. The estimated K_i of IAHQ in a spectrophotometric assay using 10-formyltetrahydrofolate (the natural diastereomer) and purified enzyme from chicken liver was found to be 94 μM. The N^9-formyl derivative (IV.286) had a K_i of 12 μM, and was therefore more potent than the parent compound. Whether this level of inhibition would suffice to cause cell growth inhibition remains to be established. An important issue in this regard is whether polyglutamates are formed intracellularly from IAHQ (or (IV.286)) and are more effective than the monoglutamates as inhibitors of the enzyme. The greater potency of (IV.286) in comparison with IAHQ was notable in view of the fact that 10-formyl-5,8-dideazafolate (K_i = 29 μM) and 5,8-dideazafolate (K_i = 24 μM) were essentially equipotent.

Growth inhibition studies with IAHQ and related compounds have been performed with a number of tumour cell lines in culture. The IC_{50} value against HCT-8 human colon adenocarcinoma cells was reported [160] to be 0.5 μM, as compared with 0.01 μM for MTX and 4 μM for 5,8-dideazafolate. Against murine L1210, P388, and L5178Y leukaemias and Walker 256 rat carcinoma, on the other hand, the IC_{50} was 5 μM, indicating a variability of as much as 10-fold, depending on the cell line. The IC_{50} of the N^9-formyl derivative (IV.286) was > 10 μM. In tests against four other human colon adenocarcinoma lines (HuTu80, HT29, SW480, WIDR), Hynes *et al.* [60] found IAHQ to have IC_{50} values of 1.7–4.5 μM, as compared with 0.6–1.1 μM for 5,8-dideazafolate and 0.009–0.025 μM for MTX. 5-Methyl-5,8-dideazaisofolate (IV.282) was slightly more potent than IAHQ against 3 of the 4 cell lines, the activity of N^9-methyl and 5,N^9-dimethyl derivatives (IV.284) and (IV.285) was in the same general range, but the activity of the N^9-formyl derivative (IV.286) (IC_{50} = 95–195 μM) was substantially lower. In assays against CEM human leukaemia lymphoblasts, Mini *et al.* [161] found (IV.286) to have an IC_{50} value of 1.5 μM, as compared with 0.015 μM for MTX. However, three MTX-resistant CEM sublines proved to be only partially cross-resistant to (IV.286). A subline (CEM/R_1) with 6.5-fold elevated DHFR activity was 100-fold resistant to MTX (IC_{50} = 1.5 μM) but only 19-fold resistant to IAHQ. Another subline (CEM/R_2) 333-fold resistant to MTX by virtue of a 5-fold increase in DHFR combined with defective MTX uptake was only 15-fold resistant to IAHQ, while a third subline (CEM/R_3) 227-fold resistant to MTX solely by virtue of impaired transport was only 12-fold resistant to IAHQ. The MTX-resistant sublines all had essentially the same TS and FPGS activity as the parent line, and their DHFR activity was equally sensitive to inhibition by MTX according to spectrophotometric assay. From these results it would appear that cross-resistance to MTX might be entirely eliminated if an analogue of IAHQ with roughly 10-fold greater potency could be found.

A CEM cell subline with 75-fold resistance to IAHQ was developed [160] and found to be as sensitive as the parent CEM line to MTX. This was consistent with the biochemical studies and strengthened the view that therapeutic strategies with TS-targeted antifolates were a viable alternative to the use of classical DHFR-targeted drugs.

Although initial *in vivo* tests with IAHQ and other members of the series against murine L1210 leukaemia were discouragingly negative, it was later discovered that IAHQ delayed tumour growth and produced a significant number of long-term survivors in mice with subcutaneously implanted colon 38 adenocarcinoma [160]. Treatment with 85 mg/kg of IAHQ on days 2 and 10 after tumour inoculation resulted in 6 of 20 mice tumour-free mice after 90 days, whereas untreated controls or mice receiving 15 mg/kg of MTX on days 2, 6, 10, and 14 were either dead or had large tumours. Activity has also been reported for IAHQ against CX-1 human colon tumour xenografts in athymic mice [162] and human osteosarcoma xenografts in newborn hamsters [163]. Against L1210 murine leukaemia, 200 mg/kg (q2d × 3) or 150 mg/kg (q2d × 5) of IAHQ produced increases in survival of only 40–50% [162]. Some increase in survival was achieved by adding 250 mg/kg of the uricosuric agent probenecid or 150 mg/kg of the calcium channel blocker verapamil to the regimen in the hope of stimulating drug retention by the tumour cells. However, these combinations proved less effective than MTX alone (136% ILS at 10 mg/kg, q2d × 3). Combinations of IAHQ and MTX were antagonistic with respect to MTX, probably because IAHQ was interfering with MTX uptake and polyglutamylation. It appears from the data published to date that future prospects for this drug may be brighter in the therapy of solid tumours than leukaemias, against which MTX is already very effective.

5,8-Dideaza compounds with N^{10} substitution

Chemistry. In 1981, Jones *et al.* [164] reported the synthesis of several 2-amino-4(3*H*)-quinazoline antifolates with substitution on N^{10}, one of which was the N^{10}-propargyl derivative (IV.287) (CB3717), also referred to in some publications as PDDF (propargyldideazafolate). This compound attracted a great deal of interest because of its unique structure, its mechanism of action, which was directed toward TS rather than DHFR, and its promising activity against tumours minimally responsive to MTX. The initial preclinical activity of CB3717 was sufficient to warrant Phase I and Phase II trials in Europe [165–168]. While the clinical performance of CB3717 has proved somewhat disappointing, this compound is nonetheless of historical importance and the biochemical strategy underlying its design [45] remains valid. Key elements

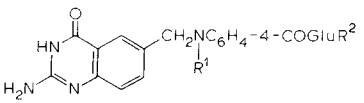
in this strategy are considered to be (a) that inhibition of TS as opposed to DHFR should spare purine, RNA, and protein synthesis, and (b) that a *folate-based* TS inhibitor ought to provide a more specific empirical test of the long-standing concept of 'thymineless death' than 5-fluorouracil and its derivatives, which undergo partial incorporation into RNA.

In the original preparation of CB3717 [164], 2-amino-6-chloromethylquinazolin-4(3*H*)-one was condensed with diethyl *N*-[4-(*N*-propargyl)aminobenzoyl]-L-glutamate, and the resulting diethyl ester ((IV.288), 28% yield) was hydrolyzed with base (81% yield). Similarly obtained from diethyl *N*-[4-(*N*-allylamino)benzoyl]-L-glutamate and diethyl *N*-[4-(*N*-propylamino)benzoyl]-L-glutamate, respectively, were the diesters (IV.289) (22% yield) and (IV.290) and the diacids (IV.291) (46% yield) and (IV.292). The UV spectra of the N^{10}-propargyl derivative [λ_{max} 229 nm (ε 50,700) 279 (23,900), 302 (26,600)] and the N^{10}-allyl derivative [λ_{max} 229 nm (ε 49,300), 276 (17,600), 312 (29,100)] showed small differences in the two longer wavelength peaks that were suggestive of a subtle stereoelectronic interaction between the π-orbitals of the allyl and propargyl groups and those of the quinazoline and/or phenyl rings. Such interactions obviously can be important in determining the conformation of a molecule and its ability to interact with an enzyme.

In a more recent paper, Jones *et al.* [169] extended this series by preparing the N^{10}-ethyl, N^{10}-isopropyl, N^{10}-cyclopropylmethyl, N^{10}-(2-fluoroethyl), N^{10}-(3-hydroxypropyl), N^{10}-(3-fluorobenzyl), N^{10}-phenacyl, N^{10}-carboxymethyl, N^{10}-(2-carboxyethyl), N^{10}-carbamoylmethyl, N^{10}-cyanomethyl, N^{10}-(3-cyanopropyl), and N^{10}-(5-uracilylmethyl) analogues (IV.293)–(IV.305). The 6-bromomethylquinazoline was used instead of the 6-chloromethylquinazoline, and alkylation reactions were conducted in *N,N*-dimethylacetamide in the presence of $CaCO_3$ (or 2,6-lutidine in some instances) as the acid scavenger. Compound (IV.297) was prepared from diethyl *N*-[4-[*N*-(3-acetoxypropyl)]-aminobenzoyl]- L-glutamate and 2-*N*-pivaloylamino-6- bromomethylquinazolin-4(3*H*)-one, a very useful intermediate because of its improved solubility. Complete deprotection of the COOH, OH, and NH_2 groups in a single step was achieved by treatment with 0.04 M NaOH at 50 °C for 18 h. The N^{10}-cyanomethyl analogue (IV.303) was prepared from di-*t*-butyl *N*-[4-(*N*-cyanomethyl)aminobenzoyl)]-L-glutamate, which offered the advantage that removal of the ester groups in the final step could be accomplished with acid to circumvent the base instability of the cyanomethyl group. The acids (IV.300) and (IV.301) were obtained by alkaline hydrolysis of the esters.

In another recent study, Caperelli and Conigliaro [170] synthesized 10-formyl-5,8-dideazafolic acid (IV.307) and 10-acetyl-5,8-dideazafolic acid (IV.308). Compound (IV.307) was obtained from 5,8-dideazafolic acid in 80%

yield by treatment with formic acid and acetic anhydride at room temperature, while (IV.308) was prepared in the same yield by reaction with acetyl chloride in DMA.

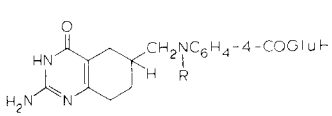


(IV.287) R^1 = $CH_2C{\equiv}CH$, R^2 = H
(IV.288) R^1 = $CH_2C{\equiv}CH$, R^2 = Et
(IV.289) R^1 = $CH_2CH{=}CH_2$, R^2 = Et
(IV.290) R^1 = *n*-Pr, R^2 = Et
(IV.291) R^1 = $CH_2CH{=}CH_2$, R^2 = H
(IV.292) R^1 = *n*-Pr, R^2 = H
(IV.293) R^1 = Et, R^2 = H
(IV.294) R^1 = *i*-Pr, R^2 = H
(IV.295) R^1 = cyclopropylmethyl, R^2 = H
(IV.296) R^1 = CH_2CH_2F, R^2 = H
(IV.297) R^1 = $CH_2CH_2CH_2OH$, R^2 = H
(IV.298) R^1 = $CH_2C_6H_4F$-3, R^2 = H
(IV.299) R^1 = $CH_2COC_6H_4$, R^2 = H
(IV.300) R^1 = CH_2COOH, R^2 = H
(IV.301) R^1 = CH_2CH_2COOH, R^2 = H
(IV.302) R^1 = CH_2CONH_2, R^2 = H
(IV.303) R^1 = CH_2CN, R^2 = H
(IV.304) R^1 = $CH_2CH_2CH_2CN$, R^2 = H
(IV.305) R^1 = 5-uracilylmethyl, R^2 = H
(IV.306) R^1 = $CH_2C{\equiv}CCH_3$, R^2 = H
(IV.307) R^1 = CHO, R^2 = H
(IV.308) R^1 = $COCH_3$, R^2 = H

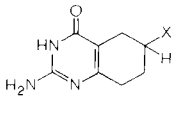
It is of interest to note that Nair *et al.* [171], in an earlier paper describing (IV.303), reported the isolation of this compound in 45% overall yield by condensation of 2-amino-6-bromomethylquinazolin-4(3*H*)-one and diethyl *N*-[4-(*N*-cyanomethyl)amino-benzoyl-L-glutamate in DMA (110 °C, 18 h), followed by alkaline hydrolysis in aqueous acetonitrile. Cyanomethylation of diethyl *N*-(4-aminobenzoyl)-L-glutamate was accomplished with bromoacetonitrile and K_2CO_3 in hot alcohol, with the monoalkylated product being isolated in 40% yield after chromatography to remove starting material and dialkylated byproduct. Jones *et al.* [169] have provided evidence that the product claimed to be (IV.303) [171] was, in fact, a mixture of (IV.303) and the corresponding amide (IV.302), an authentic sample of which proved less active than (IV.303). Nair *et al.* [171] likewise synthesized the N^{10}-cyclopropylmethyl analogue (IV.295), and in addition prepared the N^{10}-(2-butynyl) analogue (IV.306).

A 5,6,7,8-tetrahydro derivative (IV.309) of CB3717 was synthesized [172] in order to assess the importance of planarity in the B ring for TS binding. 2-Amino-6-*n*-butyloxycarbonyl-5,6,7,8-tetrahydroquinazolin-4(3*H*)-one (IV.312) was converted to the 6-bromomethyl derivative (IV.313) via the alcohol (IV.314). Condensation of (IV.313) with ethyl 4-(*N*-propargylamino)benzoate followed by alkaline hydrolysis yielded the acid (IV.315), which on mixed anhydride coupling to diethyl L-glutamate and alkaline hydrolysis was converted to (IV.309). Alternatively, (IV.313) was condensed with diethyl *N*-[4-(*N*-propargylamino)benzoyl]-L-glutamate and the product was hydrolyz-

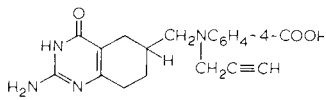
ed with base, or (IV.313) was condensed directly with *N*-[4-(*N*-propargylamino)benzoyl-L-glutamic acid. Use of *N*-(4-aminobenzoyl)-L-glutamic acid led to the N^{10}-methyl analogue, (IV.310), while reaction of (IV.313) with diethyl *N*-[4-(*N*-methylamino)benzoyl]-L-glutamate followed by hydrolysis afforded 5,6,7,8-tetrahydro-5,8-dideazafolate (IV.311).



(IV.309) R = $CH_2CH{\equiv}CH$
(IV.310) R = Me
(IV.311) R = H



(IV.312) X = CO_2Bu-*n*
(IV.313) X = CH_2Br
(IV.314) X = CH_2OH



(IV.315)

Biological activity. The first comparative data on the activity of CB3717 and its allyl and *n*-propyl analogues (IV.291) and (IV.292) as *in vitro* inhibitors of TS and DHFR from L1210 murine leukaemia cells were reported in 1981 [164]. The data immediately revealed the remarkably potent and specific effect of N^{10}-propargyl substitution on binding to TS, but not DHFR. Thus, while the K_i values of these compounds as DHFR inhibitors spanned only a narrow range (14–27 nM), their IC_{50} values as TS inhibitors varied over a 30-fold range ((IV.292), 170 nM; (IV.291), 69 nM; CB3717, 5 nM). More detailed kinetic data for the interaction of CB3717 with these enzymes were reported by Jackson *et al.* [173], who found (a) that DHFR inhibition was, as expected, competitive with dihydrofolate, and (b) that inhibition of TS from human lymphoblasts (WI-L2 cells) was competitive with 5,10-methylenetetrahydrofolate and did not increase with up to 60 min of preincubation, suggesting absence of irreversible inhibition. According to Cheng *et al.* [174], however, inhibition of TS purified from KB and HeLa cells by CB3717 is noncompetitive with respect to 5,10-methylenetetrahydrofolate. While it is possible that the mechanism of TS inhibition is not the same in all tumour cell lines, these authors proposed that the difference between their results and those of the earlier study might be related to temperature effects on the kinetics of the TS reaction.

Nair *et al.* [171] confirmed in 1983 that CB3717 was a much more potent inhibitor of TS than of DHFR in assays using bacterial enzymes. Against TS and DHFR from *L. casei*, the IC_{50} values were 0.013 and 22 μM, respectively, while against the same enzymes from *S. faecium* the corresponding IC_{50} values were 0.01 and 0.3 μM. Thus, the different effect between TS and DHFR inhibition appeared to be species-related, with the *S. faecium* enzyme probably being more predictive of the effect on mammalian enzyme. In the same

study the N^{10}-cyanomethyl analogue (IV.303) was found to be a poorer TS inhibitor than CB3717, with IC_{50} values of 0.64 μM against *L. casei* enzyme and 1 μM against *S. faecium* enzyme. Thus replacement of the acetylene group by a nitrile resulted in a 50- to 100-fold decrease in binding to TS, even though the two groups were sterically similar. However, in light of the uncertain purity of the (IV.303) used in these assays, this may have been an overestimate. According to Jones *et al.* [164], (IV.303) is only 13-fold less potent than CB3717 as an inhibitor of purified TS from L1210 cells.

Comparisons of the enzyme inhibitory and cell growth inhibitory activities of CB3717 and of the N^{10}-cyclopropylmethyl and N^{10}-(2-butynyl) analogues (IV.295) and (IV.306) were made by Nair *et al.* [171], and the superiority of the N^{10}-propargyl moiety was confirmed. While the IC_{50} values of CB3717 against *S. faecium* and *L. casei* were found to be 0.13 and 0.4 nM, respectively, the corresponding values for (IV.295) were 2.6 and 6.7 nM and those for (IV.306) were 8.1 and 86 nM. As with CB3717, however, *S. faecium* and *L. casei* strains resistant to MTX were only partly cross-resistant to (IV.295) and (IV.306).

In a recent study of the effect of reduction of ring B on the biological activity of CB3717 [172], the 5,6,7,8-tetrahydro compounds (IV.309)–(IV.311) were tested for their ability to inhibit (a) purified DHFR and TS from *L. casei*, (b) purified TS from *E. coli* and calf thymus, (c) the activity of TS in intact as well as permeabilized L1210 cells, (d) the growth of MTX-sensitive and MTX-resistant strains of *S. faecium* and *L. casei*, and (e) the growth of MCF-7 human breast carcinoma cells in culture. All three compounds were poor DHFR inhibitors, with IC_{50} values of 10–30 μM as compared with 5 μM for CB3717. Against TS from *L. casei*, the N^{10}-propargyl-5,6,7,8-tetrahydro derivative (IV.309) had an IC_{50} value of 23 μM, a value 1000-fold higher than that of CB3717, while the other two reduced compounds were even less inhibitory. Against TS from *E. coli* and calf thymus, (IV.309) gave IC_{50} values of 14 and 7 μM, respectively, while for the N^{10}-methyl analogue (IV.310) the corresponding values were 38 and 12 μM. In assays of *in situ* TS inhibition in L1210 cells permeabilized with sodium dextran sulphate (400 μg/ml, 20 min at 4 °C), IC_{50} values obtained for (IV.309) and CB3717 were 11 and 0.028 μM, respectively, a difference of 400-fold. Thus, against TS from two bacterial and two mammalian species, reduction of the ring decreased binding by two to three orders of magnitude, suggesting that the active site of TS probably has stringent spatial requirements which are best met by a planar B ring. Interestingly, when the IC_{50} values for TS inhibition by (IV.309) and the N^{10}-unsubstituted compound (IV.311) were compared in the permeabilized L1210 cell system, no difference was observed. This was in marked contrast to the

results with CB3717 and 5,8-dideazafolate in this system, which revealed a 350-fold difference in favour of the N^{10}-propargyl substitution. On the basis of these findings, the authors speculated that the mode of binding of the reduced and non-reduced quinazolines to TS might not be the same. In agreement with their decreased anti-TS activity relative to CB3717, the reduced quinazolines (IV.309) and (IV.310) were 5- to 10-fold less active than CB3717 against wild-type *S. faecium* and *L. casei* strains. However, antibacterial activity was still substantial; the IC_{50} value of (IV.310) against *S. faecium* was 0.57 nM, while that of CB3717 was only 4-fold lower. Interestingly, (IV.310) was somewhat more potent than (IV.309), suggesting that the rules for optimal N^{10} substitution in reduced and non-reduced 5,8-dideazafolates may not be the same. Growth inhibition of mammalian tumour cells, on the other hand, was not observed at concentrations as high as 50 μM. Thus, reduction of ring B was a very unfavourable structural modification where antitumour activity was concerned.

In 1986, Pogolotti *et al.* [175] published the results of a rigorous study on the kinetics and mechanism of interaction of CB3717 with TS in the presence of dUMP. Evidence was obtained for the formation of a rapidly reversible ternary complex, which undergoes slow isomerization to an isolable covalent product with Michael addition of a thiol group of the enzyme to the 5,6-double bond of the dUMP (as in the comparable reaction of TS when the nucleotide ligand is FdUMP). The complex was found to contain 2 mol of CB3717 and 2 mol of dUMP per mol of dimeric enzyme. A stable ternary complex was similarly formed when TS was incubated with CB3717 and FdUMP, but the rates of formation and dissociation of the two complexes were different. Of particular significance, the rate of dissociation of the CB3717-TS-FdUMP complex was slow. It was suggested (a) that CB3717 should be useful against tumours that do not respond well to FUra or FdUrd by reason of a low endogenous level of 5,10-methylenetetrahydrofolate (this low level would favour binding of CB3717), (b) that the slow rate of dissociation of the CB3717-TS-dUMP complex might make CB3717 especially useful in the treatment of slowly growing tumours, (c) that pretreatment with dUrd or MTX ought to potentiate CB3717 by raising dUMP levels and promoting ternary complex formation, and (d) that a combination CB3717 and FdUrd might be better than CB3717 alone against slowly growing tumours, since this would give rise to an even more stable ternary complex.

The effect of N^{10}-substitution on TS-inhibitory potency *in vitro* has been examined in detail [169]. Of the 13 representative compounds tested, only two, the N^{10}-ethyl (IV.293) and N^{10}-(2-fluoroethyl) (IV.296) analogues had IC_{50} values that were < 5-fold greater than the IC_{50} of CB3717. The IC_{50}

values of the N^{10}-(3-hydroxypropyl) (IV.297), N^{10}-(cyanomethyl) (IV.303), and N^{10}-isopropyl (IV.294) analogues were 10- to 20-fold higher; those of the N^{10}-carbamoyl (IV.302), N^{10}-carboxymethyl (IV.300), and N^{10}-(3-cyanopropyl) (IV.304) analogues were 100- to 400-fold higher; and those of the N^{10}-(2-carboxyethyl) (IV.301), N^{10}-(*m*-fluorobenzyl) (IV.298), and N^{10}-(5-uracilylmethyl) (IV.305) analogues were > 1000-fold higher. Nair *et al.* [171] reported independently that the N^{10}-cyclopropylmethyl analogue (IV.295) and the N^{10}-(2-butynyl) analogue (IV.306) had IC_{50} values of 12 and 4.4 μM, respectively, against TS, and 2.3 and 250 μM against DHFR from *L. casei*. From these results it appears unlikely that any N^{10}-substituent will be found to be superior to the propargyl group with respect to TS inhibition. However, the steric and electronic constraints in the enzyme-inhibitor complex that contribute to the remarkable specificity of this group remain unclear.

In agreement with its potent anti-TS activity, CB3717, with an IC_{50} value of 5 μM, proved more toxic than its N^{10}-propyl analogue (IV.292) (IC_{50} = 33 μM) or N^{10}-allyl analogue (IV.291) (IC_{50} = 8.8 μM) to cultured L1210 murine leukaemia cells [164]. Moreover, in assays against L1210/R71 cells, which were shown earlier to be 600-fold resistant to MTX by virtue of increased DHFR activity [176], there was only 3-fold resistance to CB3717. This compound therefore fulfilled the expectation that it would be cytotoxic irrespective of cellular DHFR content. It was also reported in this and a subsequent publication [173] that the toxicity of CB3717 to cultured L1210 cells could be prevented completely by co-incubation with 10 μM thymidine, but only partly prevented by treatment with up to 100 μM folinic acid (200-times the concentration sufficient to protect cells from the antipurine effects of MTX). This was interpreted to mean that CB3717 exerts its effects solely at the level of TS, and that only at high concentrations of leucovorin do intracellular pools of 5,10-methylenetetrahydrofolate become high enough to compete with CB3717 for the TS active site. In order to determine whether leucovorin could compete with CB3717 at the level of membrane transport, a competitive [^{3}H]MTX uptake experiment was also performed. Since MTX and reduced folates share a common transport pathway, it was likely that if MTX uptake were inhibited by CB3717, the uptake of CB3717 would be diminished in the presence of high concentrations of leucovorin. The concentration of CB3717 needed to decrease the uptake of 4 μM MTX by 50% was approximately 200 μM. It was thus possible that high levels of leucovorin could protect cells from CB3717 not only by expanding the 5,10-methylenetetrahydrofolate pool, but also by blocking transport. Interestingly, the uptake of [^{3}H]folate was inhibited about as efficiently as that of [^{3}H]MTX, suggesting that CB3717 may be an equally good substrate for the folate and reduced

folate carrier systems. Cheng *et al.* [174] have recently reported that, in contrast to the results of the Chester Beatty group [173] with L1210 cells, 100 μM folinic was able to fully protect KB cells from 1.5 μM CB3717.

Caperelli [177] has recently examined CB3717, several of the analogues from the Chester Beatty program ((IV.293)–(IV.295), (IV.298)–(IV.305)) and also the N^{10}-acetyl derivative (IV.308) as competitive inhibitors of GAR TFase, a key enzyme in *de novo* purine biosynthesis. N^{10}-Formyl-5,8-dideazafolate (IV.307), which is known to be a substrate for the enzyme [178, 179], was used as the formyl donor. The enzyme was purified from L5178Y murine lymphoma cells. While CB3717 was found to be a moderately potent inhibitor, with a K_i of 6 μM, the other members of the series varied in their K_i values from 3 μM (IV.299) to 32 μM (IV.304). By comparison, 5,8-dideazafolate had a K_i of 2 μM, while 10-acetyl-5,8-dideazafolate (IV.308) had a K_i of 1.2 μM and was therefore the most potent member of the series. It would be of interest to determine whether the polyglutamates of these compounds are more potent GAR TFase inhibitors than are the monoglutamates, as is true for other enzymes of the folate pathway. Unless polyglutamates of CB3717 prove significantly more potent than the parent drug against GAR TFase, it seems unlikely that this enzyme can be a pharmacologically important site of action in comparison with TS, which CB3717 inhibits with a K_i in the nanomolar range, even as the monoglutamate.

The activity of CB3717 against bacterial and mammalian cells was compared with that of the N^{10}-cyanomethyl analogue (IV.303) by Nair *et al.* [171]. Both compounds were also tested as inhibitors of the growth of MTX-resistant *S. faecium* and *L. casei* strains. The IC_{50} values of CB3717 against L1210 leukaemia, S180 sarcoma, and Ehrlich carcinoma cells were found to 1–2 μM as compared with 5–15 μM for (IV.303). Replacement of the carbon-carbon triple bond by the more polar carbon-nitrogen triple bond therefore produced a 5- to 10-fold decrease in potency against cultured tumour cells. Both compounds were significantly less potent than MTX, whose IC_{50} values ranged from as low as 0.00084 μM (L1210 cells) to 0.013 μM (Ehrlich cells) under identical growth conditions in low-folate medium. The IC_{50} values of CB3717 against MTX-sensitive and 2,900-fold MTX-resistant *S. faecium* were 0.25 and 36 nM, while the corresponding IC_{50} values for IV.303 were 2.0 and 360 nM. Resistance in both instances was only 100- to 200-fold. Against MTX-sensitive and > 500,000-fold MTX-resistant *L. casei*, CB3717 had IC_{50} values of 0.6 and 16,000 nM, respectively, while (IV.303) had IC_{50} values of 1.4 and > 20,000 nM. It thus appeared that both of the MTX-resistant bacterial organisms were only partly cross-resistant to the quinazolines.

Diddens *et al.* [180] examined the activity of CB3717 against several human

lymphoma cell lines with acquired resistance to MTX, and similarly demonstrated that cross-resistance between the two drugs was low. Against a 290-fold MTX-resistant Burkitt's lymphoma cell (Raji/MTX) that overexpressed DHFR 550-fold but was normal for MTX transport (as measured by the initial uptake rate), there was only 5-fold resistance to CB3717. Against a 13,000-fold MTX resistant mutant of the B-cell lymphoblastoid line WI-L2 that overproduced DHFR 110-fold and whose transport of MTX was likewise unimpaired, resistance to CB3717 was only 15-fold. Finally, against the transport-defective T-cell lymphoblastoid line CEM/MTX, whose DHFR level was normal and which was 210-fold resistant to MTX, resistance to CB3717 was only 5-fold. It thus appeared that cross-resistance was minimal not only in DHFR-overproducing cells, but also in cells whose MTX resistance seemed to be associated primarily with slow drug uptake. The most impressive results were obtained with a 200-fold MTX resistant osteosarcoma line (SAOS-2/MTX) with a marked defect in MTX influx kinetics, which showed virtually no resistance to CB3717 at all. In a more recent study [174], KB cell lines resistant to MTX by virtue of DHFR overproduction were similarly found to be only slightly cross-resistant to CB3717. For example, 2,300- and 6,700-fold resistant cells with 41- and 53-fold increases in DHFR activity were only 6- and 13-fold resistant to the quinazoline.

Preliminary *in vivo* antitumour assays with CB3717 were carried out by the Chester Beatty group [164] against L1210 leukaemia in mice. Of the several dose schedules tested the most effective by far was qd × 5, which produced 9 out of 10 long-term survivors (> 120 days) at doses of either 125 or 200 mg/kg. On this schedule, a 3 mg/kg dose of MTX produced a 71% ILS with only 2 out of 10 long-term survivors. These extremely promising results led to a decision to launch CB3717 as an alternative antifolate with possible activity against MTX-resistant tumours.

Biochemical studies with CB3717 were initially carried out in cultured human lymphoblastoid cells (WI-L2) as part of an intensive effort to bring this drug to the clinic. In addition to confirming that dThd (10 μM) protects cells from the toxicity of CB3717 (20 μM), Jackson *et al.* [173] demonstrated that protection was time-dependent, with cells tolerating a delay in dThd treatment of up to 8 h, but not 24 h. Dihydrofolate pools did not rise in the presence of a growth inhibitory concentration of drug, supporting the prediction from kinetic analysis that TS rather than DHFR should be the rate-limiting enzyme for dTMP synthesis. As expected, the dTTP pool in cells treated with CB3717 became smaller (by 88%), while the dUMP pool increased 20-fold. The dGTP pool was expanded and dATP and dCTP pools became smaller, but the changes were < 30% in either direction (though they were qualitatively differ-

ent from those induced by MTX). Ribonucleotide pools remained largely unaltered. There was also an increase in the dUDP pool, but the dUTP pool increased only slightly, suggesting that dUTP misincorporation into DNA probably did not contribute to cell growth inhibition. This was confirmed by the fact that dUrd did not increase the toxicity of CB3717, and that two inhibitors of *de novo* pyrimidine synthesis, pyrazofurin and *N*-phosphonoacetyl-L-aspartate (PALA), were non-synergistic with CB3717 in the growth inhibition assay.

The results of an *in vivo* study of the biochemical pharmacology of CB3717 in mice was reported in 1984 by Jackman *et al.* [181]. Data consistent with the view that CB3717 acts via inhibition of *de novo* dThd synthesis included the fact that normal mice treated with the drug experienced as much as a 3-fold decrease in the dThd plasma level, and that the tumour inhibitory activity of the drug at a dose of 100 mg/kg (qd × 5) in mice with L1210 leukaemia was completely abrogated by co-administration of 500 mg/kg of dThd three times a day. However, it was not clear how much the changes in plasma dThd concentrations in these animals could contribute to antitumour activity, since dThd concentrations after CB3717 administration were still high enough for salvage to be significant. Cell culture experiments revealed that the extracellular dThd concentration was a critical determinant of response to CB3717, with as little as 0.1 μM dThd being sufficient to protect the cells fully. The possibility that dUrd accumulation might modulate the action of CB3717 by interfering with the ability of the tumour to salvage and phosphorylate dThd was considered. However, a sustained increase in plasma dUrd was not observed beyond an initial rise, indicating that this mechanism is probably not a major factor. On a cautionary note, the authors pointed out that, since endogenous plasma levels of dThd in rodents are substantially higher than those in humans, the use of rodent models to evaluate the therapeutic potential of inhibitors of *de novo* dThd synthesis can be misleading.

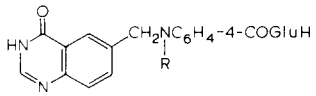
An important contributing factor to the cytotoxicity and antifolates is their ability to form non-effluxing polyglutamates inside the cell. The apparent K_m of CB3717 as a substrate for partially purified folylpolyglutamate synthetase from mouse liver was reported by Moran *et al* [94] to be 40 μM, as compared with 167 μM for MTX, 140 μM for folic acid, 56 μM for 3′,5′-dichloromethotrexate (DCM), and 18 μM for AMT. It thus appeared that CB3717 was capable of being polyglutamylated, although the distribution of polyglutamates according to chain length was not investigated. However, synthetic polyglutamates of CB3717 containing up to five glutamic acid residues were prepared [172] by a solid phase method, and their enzyme inhibitory

properties were examined. Interestingly, the addition of glutamates to CB3717 did not have nearly as great an effect on TS inhibitory potency as has been reported for MTX [182, 183]. In fact, the difference in IC_{50} between the monoglutamate and the pentaglutamate as inhibitors of TS from either *S. faecium* or *L. casei* was no more than 2-fold. Differences in dTMP synthesis in L1210 cells permeabilized for 20–40 min at 4 °C with 400 μg/ml dextran sulphate were likewise small. Similarly, there were only minor differences in IC_{50} value among the polyglutamates as inhibitors of *S. faecium* and *L. casei* DHFR. In a separate investigation [174], K_i values for the inhibition of human TS and DHFR by the polyglutamates of CB3717 were determined. While the K_i for CB3717 (IV.287) itself was 20 nM, that of (IV.287) + Glu_1 was 1.4 nM, and that of (IV.287) + Glu_3 was only 0.2 nM, a 100-fold change. Thus, while polyglutamylation of CB3717 is clearly important in preventing drug efflux from cells, there may be species differences insofar as TS inhibition is concerned.

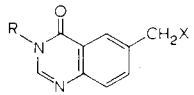
A most interesting discovery with CB3717 has been that it exhibits synergy with DHFR inhibitors, including not only MTX but also small-molecule antifolates such as trimetrexate and DDMP (metoprine) [184]. When H35 rat hepatoma cells were exposed for 72 h in culture to 0.0048 μM MTX, they grew at the same rate as untreated controls, but when they were treated concomitantly with 3.2 μM CB3717, growth inhibition increased to 73%. Treatment with 3.2 μM CB3717 and no MTX, on the other hand, produced only 31% inhibition. An even greater degree of synergy was observed when the cells were treated with MTX for only 2 h followed by 46 h of exposure to CB3717. At concentrations of MTX and CB3717 of 0.2 and 4.0 μM, respectively, growth was inhibited by 92%, whereas with the individual drugs the inhibitions observed were only 39% (MTX, 2 h) and 34% (CB3717, 46 h). Qualitatively similar results were obtained when CB3717 was combined with trimetrexate or DDMP. Tritium release assays in cells grown in the presence of [^{3}H]dUrd established that growth inhibition was commensurate with decreased TS activity. Complete protection of the cells could be achieved in all instances with 20 μM dThd. Therefore, it appeared that at low concentrations of the DHFR inhibitor which did not block purine synthesis, only TS activity was affected. The observed synergy was consistent with the hypothesis that DHFR inhibition would promote binding of CB3717 to TS by expanding the cellular dUMP pool and causing depletion of 5,10-methylenetetrahydrofolate.

In an exciting development that promises to be of theoretical as well as clinical interest, Jones *et al.* [185] recently disclosed the synthesis of a new series of CB3717 analogues (IV.316)–(IV.320) lacking a 2-amino group. These

compounds were obtained from 6-methylquinazolin-4(3*H*)-one (IV.321) by N^3-pivaloyloxymethylation and NBS bromination to give the key intermediate (IV.322), followed by condensation with *N*-propargyl or other N-substituted diethyl *N*-(*p*-aminobenzoyl)-L-glutamates, and finally alkaline hydrolysis. The rationale for the synthesis of these 2-de(amino) compounds appears to have been to increase water solubility while retaining at least some binding affinity for TS. The expected increase in water solubility was realized, but, as summarized below, the cellular pharmacologic properties of these compounds proved even more remarkable.



(IV.316) R = H
(IV.317) R = Me
(IV.318) R = Et
(IV.319) R = $CH_2CH{=}CH_2$
(IV.320) R = $CH_2C{\equiv}CH$

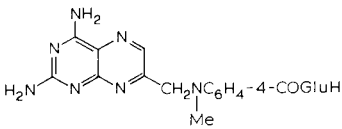


(IV.321) R = X = H
(IV.322) R = Me_3COCH_2, X = Br

In assays against TS from L1210 cells [185], the N^{10}-unsubstituted analogue (IV.316) had an IC_{50} of 23 μM, the N^{10}-methyl, -ethyl, and -allyl analogues (IV.317)–(IV.320) had IC_{50} values of 0.5–1.1 μM, and the N^{10}-propargyl analogue (IV.320) ('2-deamino-CB3717') had an IC_{50} value of 0.16 μM. The K_i of (IV.320) against DHFR from rat liver was 30-fold higher than that of CB3717. Thus, while removal of the 2-amino group from CB3717 resulted in an 8-fold loss of inhibitory potency against the primary target enzyme, there was an even greater decrease in potency against DHFR. On this basis, the authors predicted that (IV.320) might be a more selective TS inhibitor than CB3717. While a decrease in TS inhibitory activity might be expected to lead to decreased cytotoxicity, the opposite was observed. The IC_{50} value of (IV.320) was found to be 0.4 μM, a concentration 8-fold lower than that of CB3717. This surprising result was ascribed tentatively to improved membrane transport. It should be noted that at this early stage in the development of the 2-de(amino) series, the question of whether N^{10}-propargyl substitution is optimal for antitumour activity remains open. For example (IV.320) and the N^{10}-unsubstituted 2-de(amino) analogue had the same IC_{50} value against L1210 cells, even though the latter compound was 140-times more active as an inhibitor of purified TS.

Attachment at C^7 of the bridge

While the possibility that MTX prepared via the Waller reaction was a 7- rather than 6-substituted pteridine derivative was dismissed early, it remained of at least academic interest to determine whether '7-MTX' (IV.323) has biological activity. Suster *et al.* [186] described a synthesis of this compound starting from 2,4-diamino-7-chloromethylpteridine, which was prepared in 80% yield from 2,4,5,6-tetraaminopyrimidine and 1,1,3-trichloroacetone at pH 5.5–6.0. The control of pH was found to be critical, since a mixture of 6- and 7-isomers was formed when the reaction was performed below pH 5. Condensation of the pteridine with *N*-(4-methylamino)benzoyl-L-glutamic acid in NaOAc buffer at pH 3.0–3.5 for several hours, with gradual increase of the reaction temperature from 45 to 75 °C afforded (IV.323) in 12–16% yield. The UV spectrum [λ_{max} (0.1 M HCl) 312 nm (ε 24,100)] was distinguishable from that of MTX, whose maximum in 0.1 M HCl was at 306 nm. The NMR spectrum in d_6-DMSO showed the C-6 proton as a singlet at δ 8.51, whereas the C-7 proton in MTX gave rise to a singlet at δ 9.01.

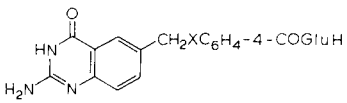


(IV.323)

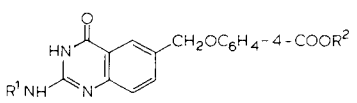
In vivo experiments confirmed that '7-MTX' (IV.323) was much less active than MTX [186, 187]. The LD_{50} value (single dose) in rats was 690 mg/kg whereas that of MTX was 18 mg/kg, and in L1210 leukaemic mice only a 30% ILS was achieved at 200 mg/kg (qd × 8) whereas with 0.3 mg/kg of MTX, on a qd × 10 schedule, the ILS was 130%. In assays against Walker 256 carcinoma, 50 mg/kg (qd × 13) gave only a 33% inhibition of tumor growth relative to controls, whereas a single 5 mg/kg dose of MTX sufficed to inhibit tumour growth by 80%. In view of the very large difference in activity between the two compounds, it is certainly possible that these data actually reflected the presence of a trace amount of MTX (0.1–0.2%) in the sample. It is unknown at present whether the lack of *in vivo* activity of (IV.323) is a consequence of weak DHFR binding, poor cellular uptake, lack of polyglutamylation, or all of the above. It may be noted that '7-folic acid' was synthesized in 1952 by the Lederle group [188] and found to be devoid of biological activity.

Miscellaneous changes in regions B and C

Analogues of 5,8-dideazafolic acid with N^{10} replaced by O or S to give 5,8-dideaza-10-oxafolic acid (IV.324) and 5,8-dideaza-10-thiafolic acid (IV.325), respectively, were synthesized in 1977 by Oatis and Hynes [155]. For the synthesis of (IV.324), 6-bromomethyl-2-pivaloylaminoquinazolin-4(3*H*)-one was condensed with methyl 4-hydroxybenzoate in the presence of $CsHCO_3$ and the resulting ester amide (IV.326) was hydrolyzed sequentially with acid and base to obtain the acid (IV.327). Mixed anhydride coupling of (IV.327) in DMSO-THF solution with Merrified resin-linked α-benzyl L-glutamate, followed by treatment with base to detach the product from the resin and cleave the benzyl ester, afforded (IV.324) in 60% yield (based on the amount of (IV.327) consumed). For the synthesis of (IV.325), unprotected 2-amino-6-bromomethylquinazolin-4(3*H*)-one was condensed with diethyl *N*-(4-mercaptobenzoyl)-L-glutamate and the resultant ester was hydrolyzed with 0.2 M NaOH.



(IV.324) X = O
(IV.325) X = S

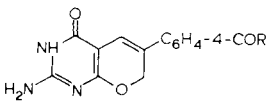


(IV.326) R^1 = Me_3CCO, R^2 = Me
(IV.327) R^1 = R^2 = H

In assays against cultured HCT-8 human colon adenocarcinoma cells, (IV.324) was found to have an IC_{50} of 0.2 μM as compared with 4 μM for 5,8-dideazafolic acid and > 10 μM for 5,8,10-trideazafolic acid [160]. The 10-thia analogue (IV.325) had an IC_{50} of 3 μM. Thus, replacement of N^{10} by O led in this cell line to a > 20-fold increase in potency. Replacement by S had only a small effect, but was nonetheless superior to replacement by CH_2. When (IV.324) and (IV.325) were tested *in vivo* against L1210 leukaemia in mice, significant activity was unfortunately not observed [155]. It should be noted, however, that the compounds were given only as a single dose, and that the highest dose tested was only 150 mg/kg. Since toxicity was not observed, it would have been appropriate to increase the dose and also use a multidose schedule (e.g., qd × 9). Further studies of these analogues might be of interest, particularly with respect to their potential effect on folate-requiring enzymes of the *de novo* purine pathway. In a recent study [62], (IV.324) and (IV.325) were found to be inhibitors of AICAR TFase, the last enzyme in the *de novo* biosynthetic pathway to purine nucleotides. The estimated K_i for inhibition of the reaction between AICAR and the natural diastereomer of 10-formyltetra-

hydrofolate was found to be 2.9 μM for (IV.324) and 27 μM for (IV.325). The difference in potency between the O and S analogues as AICAR TFase inhibitors was similar to the difference in potency of these compounds as cell growth inhibitors, suggesting a possible cause-and-effect relationship.

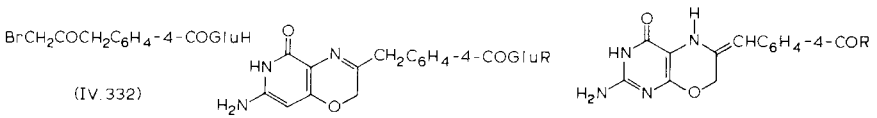
Novel examples of 7,8-dihydrofolate analogues in which the NH at position 8 is replaced by O and the bridge region is either shortened by N^{10} deletion or eliminated altogether were synthesized in 1983 by Nair *et al.* [189]. Condensation of 2,5-damino-4,6-dihydroxypyrimidine and 4-methoxycarbonylphenacyl bromide gave the ester (IV.328) (94%), which on alkaline hydrolysis formed the acid (IV.329) (76%). Mixed anhydride coupling (i-BuOCOCl-*N*-methylmorpholine) of (IV.329) with diethyl L-glutamate in DMSO solution followed directly by alkaline hydrolysis of ester (IV.330) afforded a 60% yield of a product formulated as *N*-[(2-amino-4(3*H*)-oxo-7,8-dihydro-8-oxa-6-pteridinyl)benzoyl]-L-glutamic acid (IV.331). The NMR spectrum of (IV.331) in trifluoroacetic acid solution showed the CH_2 protons at position 7 as a singlet at δ 5.5. The UV spectrum in 0.1 M NaOH showed maxima at 247 and 375 nm.



(IV.328) R = Me
(IV.329) R = H
(IV.330) R = Et
(IV.331) R = H

In a similar vein, condensation of 2,5-diamino-4,6-dihydroxypyrimidine with diethyl *N*-[4-(1-bromo-2-oxopropyl)benzoyl)-L-glutamate (IV.332) in the presence of MgO as an acid scavenger, followed by alkaline hydrolysis (0.1 M NaOH in deoxygenated MeCN-H_2O, 6 h at 25 °C) gave the diester (IV.333) (73%) and *N*-[[(2-amino-4(3*H*)-oxo-7,8-dihydro-8-oxa-6-pteridinyl)methyl]-benzoyl]-L-glutamic acid (IV.334) (30%), respectively [184]. The low yield in the hydrolysis was found to be due to the existence of another product identified as *N*-[(2-amino-4(3*H*)-oxo-7,8-dihydro-8-oxa-6-pteridinyl)methenyl]benzoyl-L-glutamic acid (IV.335). In an alternative scheme developed to confirm the structure of (IV.335), 2,5-diamino-4,6-dihydroxypyrimidine was condensed with methyl 4-(1-bromo-2-oxopropyl)benzoate and the ester group was cleaved with base to obtain the acid (IV.336) in a combined two-step yield of 35%. Mixed anhydride coupling to diethyl *N*-glutamate followed directly by alkaline hydrolysis then gave (IV.335) in addition to some (IV.334). The NMR spectrum of (IV.334) in trifluoroacetic acid showed the bridge CH_2 protons

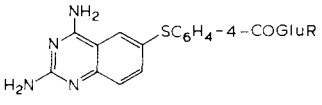
as a singlet at δ 5,5, the ring CH_2 protons at position 7 as a singlet at δ 4.41, and the phenyl protons as a pair of doublets at δ 7.6 and 7.98. The UV spectrum in 0.1 M NaOH showed peaks at 245, 275 and 325 nm. The NMR spectrum of the isomer (IV.335) in trifluoroacetic acid showed the bridge CH_2 protons as a singlet at δ 5.5 and the phenyl protons as a pair of doublets at δ 7.75 and 7.9, but lacked a signal in the δ 4.0–5.0 region that could be assigned to ring CH_2 protons. The UV spectrum in 0.1 M NaOH showed peaks at 250 and 400 nm, and was thus easily distinguishable from that of the endocyclic double-bond isomer (IV.334).



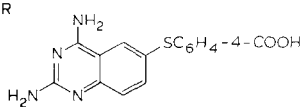
Interest in the biological properties of compounds such as (IV.331) and (IV.334) stems from the fact that they may be viewed as structural analogues of dihydrofolate that cannot undergo enzymatic oxidation to the fully aromatic form. While it would perhaps have been more useful in this regard to examine analogues with an intact CH_2NH bridge, bioassays of (IV.331) and (IV.334) were nonetheless undertaken [189]. Unfortunately, neither compound inhibited *L. casei* growth at concentrations of up to 10 μM, or showed any inhibition of *L. casei* TS at concentrations of up to 100 μM. Extremely marginal activity (IC_{50} = 3.3 μM) was shown by (IV.334) against *S. faecium*. It would appear that further work in this series would not be productive unless it were possible to synthesize compounds with at least two atoms in the bridge.

Another example of the simultaneous modification of regions B and C of the classical antifolate structure was provided in 1980 by Werbel *et al.* [190] in the form of the 2,4-diaminoquinazoline-6-thioether derivative (IV.337). Access to this compound was gained by condensation of the acid (IV.339) with diethyl L-glutamate in the presence of DCC, pentachlorophenol and hydroxybenzotriazole to form the diester (IV.338), followed by alkaline hydrolysis. The yield in the coupling reaction was only 13%, indicating that DCC is not a very useful reagent for this purpose. Compound (IV.339) was prepared in a two-step yield of 90% from the amino nitrile (IV.340) by annulation with guanidine and alkaline ester hydrolysis. The IC_{50} value of (IV.337) as an inhibitor of DHFR from rat liver was 200 nM, showing that shortening of the

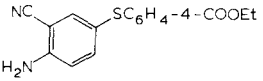
bridge had dramatically decreased binding to this enzyme. Interestingly, however, (IV.337) was a good inhibitor of DHFR from *Trypanosoma cruzi*, with an IC_{50} value of 0.5 nM. While this 400-fold selectivity between human and trypanosomal DHFR suggested that (IV.337) might be therapeutically useful against parasitic diseases, activity against malaria *in vivo* was not observed.



(IV.337) R = H
(IV.338) R = Et

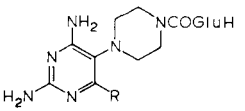


(IV.339)



(IV.340)

An even more dramatic example of the concomitant modification of regions B and C was provided recently in the form of the 2,4-diamino-5-(4-arylpiperazino)pyrimidines (IV.341)–(IV.344) [191]. These compounds may be viewed as hybrid molecules combining structural features of the classical antifolates with those of non-classical 2,4-diaminopyrimidine antitumour antifolates. Potent inhibition of DHFR from WI-L2 human lymphoblasts was observed with a number of the compounds in this series, including some in which the glutamate moiety was either modified (for example, by esterification) or replaced by smaller substituents. For the 6-methyl analogue (IV.342), the K_i was found to be 53 pM as compared with 3 pM for MTX and 770 pM for metoprine. An interesting feature of compounds (IV.341)–(IV.344) was that they were accepted, albeit to varying degrees, as substrates for folylpolyglutamate synthetase. The best substrate was the 6-unsubstituted analogue (IV.341) (K_m = 16.7 $\pm$ 2.8 μM). The relative efficiency of polyglutamylation of this compound (V_{max}/K_m) was 10- to 15-fold greater than that of folic acid or MTX and was about the same as that of AMT. Introduction of 6-alkyl substituents increased the K_m by as much as 120-fold but decreased the relative V_{max} only slightly. The effect on K_m appeared to be related to substituent chain-length.



(IV.341) R = H
(IV.342) R = Me
(IV.343) R = Et
(IV.344) R = *n*-Pr

In assays of growth inhibition against antifolate-resistant cell lines [191], (IV.341) was found to be only partially cross-resistant with either MTX or the lipid-soluble DHFR inhibitor, trimetrexate. Against a 50-fold DHFR-overproducing WI-L2 human lymphoblast subline with 147-fold resistance to MTX, resistance to (IV.341) was 54-fold as compared with 47-fold for trimetrexate. Similarly, against a different WI-L2 subline in which resistance had been induced with trimetrexate to a level of 62-fold, only 37-fold resistance to (IV.341) was observed. This cell line owed its trimetrexate resistance to decreased drug accumulation but remained MTX-sensitive, indicating that resistance was not associated with DHFR overproduction. Finally, in assays against a subline of L1210 murine leukaemia in which 273-fold MTX resistance had been induced by stepwise selection in the presence of CI-920 (an inhibitor of transport), resistance to (IV.341) was only 21-fold. In these cells, DHFR levels were normal, but MTX influx was impaired. Qualitatively similar patterns of low cross-resistance were observed with a number of other congeners. This novel class of antifolates therefore seems to merit further study.

Reported data on the *in vivo* antitumour activity of these novel compounds have been promising [191]. When administered qd × 5 starting on day 3 following tumour implantation (a more stringent test than treatment starting on day 1), a low dose of (IV.341) of just 0.8 mg/kg was sufficient to produce a 46% ILS in mice with L1210 leukaemia. The 6-methyl analogue (IV.342), when given qd × 5 at a dose of 50 mg/kg starting 1 day post-implantation, gave a 122% ILS. It should be noted, however, that these glutamate derivatives were not actually the most efficacious members of the series. In fact, certain of the analogues with smaller *para* substituents, such as CHO, COOH, $CONH_2$, $COCH_3$, CO_2Et, SO_3H, and SO_2NMe_2, on the phenyl ring were therapeutically superior to the glutamates, although higher dosages had to be used.

CHANGES IN REGION D

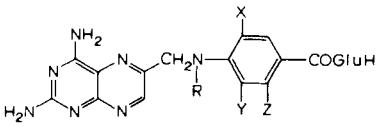
Substitution on the phenyl ring of antifolates was described as early as 1951 by Cosulich *et al.* [192] in the form of 3′-chloroAMT (V.1), 3′-bromoAMT (V.2), and 3′-chloroMTX (V.3), which were obtained by chlorination or bromination of the parent compounds in AcOH. Other 3′-chloro compounds reported by the Lederle group [192] were the N^{10}-nitroso, 9,10-dimethyl ('adenopterin'), 2-*N*,*N*-dimethylamino and 4-piperidino analogues, as well as several with side-chain moieties other than glutamic acid. Compound (V.3)

and 3′-chloroadenopterin displayed significant activity in antimicrobial assays, and were more potent than either (V.1) or (V.2) when compared with 10-methylfolic acid as a standard. Chlorination of folic acid at the 3′ and 5′ positions was also reported [192]. In a subsequent publication, Angier and Curran [193] reported that MTX could be converted to either the 3′-chloro derivative (V.3) or to 3′,5′-dichloroMTX ((V.4), DCM) with Cl_2/$HCONH_2$. The optimal conditions for obtaining (V.4) employed 6.5 mol Cl_2/mol of MTX, and the authors suggested that the active chlorinating agent was probably *N*-chloroformamide. Interestingly, Br_2-$HCONH_2$ failed to brominate either MTX or (V.3). however 3′-bromoMTX (V.5) was obtained in 80% yield with Br_2 in 6 M HCl, while chlorination of (V.5) with Cl_2/$HCONH_2$ afforded 3-bromo-5′-chloroMTX ((V.6), 50% yield). The 3′,5′-dibromo compound could not be prepared. Comparison of UV spectra revealed a small bathochromic displacement in the long-wavelength maximum of (V.3) [λ_{max} (0.1 M NaOH) 257 nm (ε 27,600), 280 sh (18,600), 362 (8,400)] in comparison with (V.4) [λ_{max} (0.1 M NaOH) 258 nm (ε 25,600), 370 (7,600)] which appeared to be related to increased twisting of the 3′,5′-disubstituted phenyl ring. Interestingly, the UV spectrum of the 3′-bromo analogue (V.5) resembled that of (V.3) rather than (V.4), suggesting that a single bromine atom *ortho* to N^{10} brought about a degree of twisting comparable to the effect of two smaller *ortho* chloro substituents. Bromination of the phenyl ring in AMT has been claimed to occur at both the 3′ and 5′ position, giving (V.7) [142, 144]. Lack of N^{10}-methyl substitution in AMT might be expected to facilitate dibromination of the molecule in comparison with MTX. N^{10}-Ethyl substitution does not seem to impede dichlorination, as Montgomery *et al.* [14] have shown that treatment of N^{10}-ethylAMT (IV.1) with Cl_2 in 6 M HCl affords the 3′,5′-dichloro derivative (V.8). An important side-reaction in MTX chlorination reactions has been found to be oxidative C^9–N^{10} bond cleavage [194]. An improved method of preparation of (V.3) and (V.4) from MTX and of 3′,5′-dichlorofolic acid from folic acid using *t*-BuOCOCl in AcOH at room temperature was developed by Martinelli and Chaykovsky [195], but chlorination of AMT by this process was not described. Chlorination of homofolic acid was described in 1974 by Lee *et al.* [196], who carried out the reaction in 6 M HCl.

Introduction of substituents other than Cl, Br, I and NO_2 was accomplished by the Lederle group [197] via a Waller synthesis using 2,4,5,6-tetraaminopyrimidine, 2,3-dibromopropionaldehyde, and appropriately substituted *N*-(2′- or 3′-X-4-aminobenzoyl)-L-glutamic acids (or their diethyl esters). The latter were prepared from the corresponding 4-nitro derivatives by reduction with Zn(Cu). Compounds obtained in this manner were the 3′-methyl and 3′,5′-dimethyl analogues, (V.9) and (V.10), of AMT. Tocumfcik and Seeger [198]

subsequently used this process to also prepare the 3′-fluoro and 3′,5′-fluoro analogues (V.11) and (V.12) of MTX. The requisite *N*-(4-*N*-methylamino-3-fluoro)- and *N*-(4-*N*-methylamino-3,5-difluoro)benzoyl-L-glutamic acids were obtained from the corresponding 4-iodo compounds by reaction with methylamine. More recently, the 2′- and 3′-fluoro derivatives (V.13) and (V.14) of AMT were prepared by Henkin and Washtien [199] by condensation of the appropriate di-*t*-butyl *N*-(4-aminobenzoyl)-L-glutamate intermediates with 2,4-diamino-6-bromomethylpteridine, followed by acidolysis of the ester groups. The UV spectra of (V.11) [λ_{max} (0.1 M HCl) 243 nm (log ε = 4.25), 290 (4.21), 330 sh (4.07); λ_{max} (0.1 M NaOH) 225 nm (log ε = 3.25), 259 (4.46), 368 (3.90)] and (V.12) [λ_{max} (0.1 M HCl) 243 nm (log ε = 4.32), 290 (4.16), 334 (4.10); λ_{max} (0.1 M NaOH) 225 nm (log ε = 4.33), 259 (4.48), 370 (3.91)] were more nearly alike than those of the chloro analogues (V.3) and (V.4), in agreement with the smaller size of F in comparison with Cl. That the effect of halogen substitution is not determined solely by steric bulk, however, is suggested by the fact that the long-wavelength maximum in 0.1 M NaOH spectrum of the 3′-fluoro analogue (V.13) is closer to that of 3′-bromoMTX (V.5) than it is to the spectrum of (V.3).

The Lederle group reported briefly that AMT was iodinated with iodine monochloride to a product believed to be the 3′,5′-diiodo derivative, and was converted to 3′,5′-dinitro derivative (V.15) with HNO_3-H_2SO_4 at 0 °C [197]. Loo *et al.* [200], however, subsequently found, that iodination of AMT with iodine monochloride in DMF gave a 38% yield of 3′-iodoAMT (V.16), along with a number of other products including 2,4-diaminopteridine-6-carboxaldehyde, but no trace of the 3′,5′-diiodo derivative. Iodination of folic acid has similarly been found to occur only at the 3′-position [201]. A modified iodination procedure involving addition of AMT and non-labelled



(V.1) R = H, X = Cl, Y = Z = H
(V.2) R = H, X = Br, Y = Z = H
(V.3) R = Me, X = Cl, Y = Z = H
(V.4) R = Me, X = Y = Cl, Z = H
(V.5) R = Me, X = Br, Y = Z = H
(V.6) R = Me, X = Br, Y = Cl, Z = H
(V.7) R = H, X = Y = Br, Z = H
(V.8) R = Et, X = Y = Cl, Z = H
(V.9) R = H, X = Me, Y = Z = H
(V.10) R = H, X = Y = Me, Z = H
(V.11) R = Me, X = F, Y = Z = H
(V.12) R = Me, X = Y = F, Z = H
(V.13) R = H, X = F, Y = Z = H
(V.14) R = H, X = Y = H, Z = F
(V.15) R = H, X = Y = NO_2, Z = H
(V.16) R = H, X = I, Y = Z = H
(V.17) R = H, X = N_3, Y = Z = H

iodine and iodic acid in DMF/CCl_4 to aqueous sodium [^{131}I]iodide was developed by Johns *et al.* [202] to prepare 3′-[^{131}I]iodoAMT for organ distribution studies in animals and for external tumour scanning in humans. The 3′-iodo derivative of MTX was not described in these papers.

The most recent introduction of a non-halogen substituent in the phenyl ring was accomplished by Holmes *et al.* [203] to obtain 3′-azidoAMT (V.17). 2-Nitro-4-(*N*-fluorenylmethoxycarbonyl)aminobenzoic acid was successively reduced with sodium dithionite, diazotized and treated with NaN_3, converted to an acid chloride, condensed with dimethyl L-glutamate, and N-deprotected with pyrrolidine to obtain dimethyl *N*-(4-amino-3-azidobenzoyl)-L-glutamic acid. Coupling to 2,4-diamino-6-bromomethylpteridine followed by alkaline hydrolysis of the ester groups then gave (V.17) in approximately 10% overall yield. The product showed the expected UV absorption features [λ_{max} (pH 7.2) 257 nm (ε 30,500), 280 (17,400), 373 (6,650)], but the spectrum had to be determined in a special rapid-scanning instrument, as irradiation at 300 nm led to 90% photolysis to a complex mixture of products within 20 s. The 3′-azido compound was an inhibitor of bacterial DHFR, giving a K_i of 0.03 and 0.16 nM against enzyme from different strains of *E. coli*, but was less potent than AMT (K_i 0.006 nM). Activity against TS was also determined and found to be comparable to that of AMT; however, as is typically true of classical antifolates of the 2,4-diaminopteridine family, inhibition of TS required concentrations several orders of magnitude higher than those for DHFR inhibition. The synthesis of (V.17) was prompted in part by a desire to examine its potential as a photoaffinity ligand, but unfortunately this hope was only minimally fulfilled. Photolysis of the DHFR-[^{3}H]N_3AMT complex for up to 10 min led to only 3.5% covalent binding, which appeared to represent merely 'pseudophotoaffinity labelling', since it was no greater in the absence of light. Binding also seemed not to be active-site-specific, since it was not diminished in the presence of MTX and since enzyme activity was unaffected by photolysis in the presence or absence of drug. According to the authors, the disappointing inability of (V.17) to serve as a DHFR photoaffinity label is due to the fact that, when the molecule binds to the active site, the azido group lies too far away from any potential reactive sites on the enzyme and therefore reacts mainly with water during the photolysis.

With the exception of the 3′,5′-dichloro derivative (V.4) (DCM), which showed promising preclinical activity [204] and was eventually moved to clinical trial [205, 206], the biochemical and biological properties of phenyl ring substituted analogues of MTX and AMT have been examined rather sparingly. In a comparative study of the *in vitro* biological activity of DCM and MTX [21], the 3′,5′-dichloro derivative was found to have an IC_{50} value of

0.02 μM against *S. faecium* (versus 0.004 μM for MTX), an IC_{50} of 0.001 μM against DHFR from L1210 cells (versus 0.0015 μM for MTX), and IC_{50} values of 0.013 and 0.017 μM against P388 mouse leukaemia cells and CEM human lymphoblasts, respectively (versus 0.022 and 0.040 μM for MTX). *In vitro*, therefore, DCM appears to be more potent than MTX, even though a substantially larger dose needs to be given *in vivo* to elicit antitumour activity. An important difference between DCM and MTX at the pharmacological level is that DCM is more efficiently extracted by the liver [207], where it is converted by aldehyde oxidase to the much less active compound 7-hydroxy-DCM [208, 209]. Because of this extensive detoxification, the therapeutic dose of DCM is much higher than that of MTX [204–206]. A further difference between DCM and MTX is that, while MTX is cleared mainly through the kidneys, the dominant clearance route for DCM is hepatobiliary. This property has recently been used as the basis for a clinical protocol in which patients received a combination of *cis*-diamminedichloroplatinum(II) and DCM rather than MTX with a view to minimizing renal toxicity [210]. The N^{10}-ethyl-3′,5′-dichloro derivative (V.8) was reported to inhibit pigeon liver DHFR with an IC_{50} value of 0.050 μM versus 0.045 μM for MTX and the growth of KB cells with an IC_{50} value of 0.022 μM versus 0.004 μM for MTX [14]. *In vivo*, (V.8) produced a 70% ILS when given at a dose of 100 mg/kg (qd × 9) to L1210 leukaemic mice. Thus, replacement of the N^{10}-Me group by N^{10}-Et in DCM appeared to be well tolerated in terms of DHFR binding and the ability to inhibit tumour cell growth.

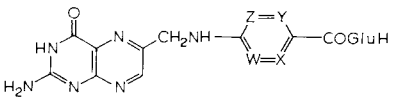
3′,5′-DibromoAMT (V.7) has been reported to be a good inhibitor of DHFR, with an IC_{50} value comparable to, or greater than, that of AMT [147, 148]. Activity was also assayed against enzymes of the folate pathway other than DHFR, but was substantially lower. *In vivo*, (V.7) showed a broader therapeutic dose range and produced greater survival rates than either MTX or AMT when administered to mice with AKR leukaemia [149, 150]. The 3′,5′-dibromo compound also seemed to have a higher therapeutic index than MTX or AMT and to be much less toxic when given on a chronic schedule. The 5,6,7,8-tetrahydro, 5-formyl-5,6,7,8-tetrahydro, and 5,10-methylene-5,6,7,8-tetrahydro derivatives of (V.7) were studied as well, but were not better than the parent compound.

The biochemical and biological activities of the 2′- and 3′-fluoro derivatives (V.13) and (V.14) of AMT have been described by Henkin and Washtien [199]. In assays against *E. coli* and *L. casei* DHFR, the K_i values of (V.13) were 0.08 and 0.095 nM, respectively, while those of (V.14) were 0.03 and 0.06 nM. The corresponding values for AMT were 0.09 and 0.11 nM. Thus, a 3′-fluoro substituent appeared to increase DHFR affinity some 2- to 3-fold,

whereas a 2′-fluoro substituent (further away from N^{10}) had almost no effect. The same trend was evident in assays using DHFR from HeLa cells. When the 2′- and 3′-fluoro derivatives were tested as inhibitors of L1210 cell growth in culture, they gave IC_{50} values of 2.3 and 1.0 nM, respectively, as compared with 1.8 nM for AMT. Against HuTu80 human colon carcinoma cells, the corresponding IC_{50} values were 4.8 nM (V.13), 2.3 nM (V.14), and 4.7 nM (AMT). Thus, the fluoro compounds obeyed the same order with respect to cytotoxicity as they did with respect to DHFR binding. Although *in vivo* antitumour activity data were not reported for these compounds, two other ring-fluorinated compounds, the 3′-fluoro and 3′,5′-difluoro analogues (V.11) and (V.12), are claimed [198] to have activity against 6C3HED mouse lymphosarcoma comparable to that of DCM.

An interesting rationale for structural modification of the phenyl ring in folates was put forward in 1971 by Roberts and Shealy [211]. According to this rationale, the ability of folate cofactors to serve as one-carbon acceptors or donors should be influenced by the electron availability at N^{10}. It was postulated that a decrease in electron availability would diminish the ability of the molecule to form one-carbon donor species (for example a 5,10-methylene derivative), while an increase in electron availability would enhance the stability of such species and thus render more difficult the transfer of a one carbon fragment from the donor species to the acceptor (for example dUMP). While this interesting biochemical concept did not culminate in the development of useful new drugs, it nonetheless led to the synthesis of a number of novel folate and antifolate analogues modified in Region D.

In the first attempt to test the concept of electron availability at N^{10}, 2′-azafolic acid (V.18) and 3′-azafolic acid (V.19) were synthesized [211]. 5-Nitropicolinic acid was sequentially condensed with diethyl L-glutamate in the presence of DCC, and reduced catalytically with Raney nickel at atmospheric pressure to give an amine. Condensation of the amine with 2-acetamido-4(3*H*)-oxopteridine-6-carboxaldehyde in DMSO in the presence of molecular sieves for 4 days yielded a Schiff's base, which on reduction

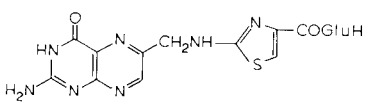


(V.18) W = Y = Z = CH, X = N
(V.19) W = N, X = Y = Z = CH
(V.20) W = Z = CH, X = Y = N
(V.21) W = Z = N, X = Y = CH
(V.22) W = X = N, Y = Z = CH

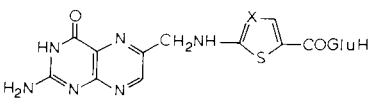
($NaBH_4$-DMF), hydrolysis of the amide and ester blocking groups (0.1 M NaOH), and reoxidation of the pyrazine moiety (30% H_2O_2), was converted to (V.18) in approximately 5% overall yield. Reduction of the Schiff's base with toluenethiol instead of $NaBH_4$ gave a better overall yield and obviated the need for H_2O_2 oxidation, since unwanted reduction of the pyrazine ring was no longer a problem. For the synthesis of (V.19), 6-aminonicotinic acid was condensed with diethyl L-glutamate by the mixed anhydride or carbodiimide route, and the product was coupled to 2-acetamido-4(3*H*)-oxopteridine-6-carboxaldehyde in the presence of toluenethiol. Alternatively, 6-aminonicotinic acid or ethyl 6-aminonicotinate was elaborated to N^2,N^{10}-diacetyl-3′-deazapteroic acid, the latter was condensed with diethyl L-glutamate, and 0.1 M NaOH was used to remove amide- and ester-protecting groups at the same time. NMR spectra of (V.18) and (V.19) in trifluoroacetic acid showed the C-7 proton as a singlet at δ 9.05. As expected, the CH_2N bridge protons gave a resonance signal at lower field in the 3′-deaza analogue (V.19) (δ 5.18) than in the 2′-deaza analogue (V.18) (δ 5.05). The UV spectra of the two compounds [(V.18): λ_{max} (0.1 M HCl) 228 nm (ε 25,900), 291 (18,600), 333 (16,700); λ_{max} (pH 7) 281 nm (ε 26,300), 305 sh; λ_{max} (0.1 M NaOH) 255 nm (ε 24,900), 283 (22,300), 304 sh, 363 (9,250). (V.19): λ_{max} (0.1 M HCl) 261 nm (ε 24,800), 316 (15,500); λ_{max} (pH 7) 277 nm (ε 32,000), 347 (8,020); λ_{max} (0.1 M NaOH) 258 nm (ε 28,200), 275 (29,900), 365 (8,900)] revealed a marked difference between the 2′- and 3′-aza compounds in the position of the two longest-wavelength bands at acid pH. The displacement of these bands toward the blue end of the spectrum in the 3′-aza compound was indicative of an electron-withdrawing effect that would undoubtedly modify the ability of N^{10} to serve as a one carbon acceptor. Unfortunately, neither (V.18) nor (V.19) proved to be of much interest except for the fact that both compounds had the ability to support the growth of *S. faecium*. No inhibition of mammalian cell growth was observed, and there was no activity against L1210 leukaemia in mice at doses of up to 48 mg/kg (qd × 9) of (V.18) and 75 mg/kg (qd × 9) of (V.19).

In another attempt to generate an antifolate by altering the electron density at N^{10}, Roberts and Shealy [212] synthesized and tested the thiazole analogues (V.23) and (V.24). The method of preparation generally followed the scheme used to form the pyridine analogues, and again gave low overall yields. The NMR spectrum of the 4-substituted thiazole (V.23), taken in trifluoroacetic acid, showed the CH_2N bridge protons as a singlet at δ 5.20, while the spectrum of the 5-substituted thiazole (V.24) showed these protons at δ 5.15, suggesting lower electron density at N^{10} in the former structure. Interestingly, there was an opposite shift to higher field for the C-7 proton in (V.23) (δ 9.00)

relative to (V.24) (δ 9.05). The UV spectra of the two compounds [(V.23): λ_{max} (0.1 M HCl) 250 nm (ε 22,400), 322 (8,700); λ_{max} (pH 7) 234 nm (ε 24,300), 275 (20,300), 348 (7,020); λ_{max} (0.1 M NaOH) 255 nm (ε 32,600), 365 (8,350). (V.24): λ_{max} (0.1 M HCl) 250 nm (ε 15,200), 281 (20,600), 320 (9,650); λ_{max} (pH 7) 279 nm (ε 18,800), 302 (20,800), 342 (7,920); λ_{max} (0.1 M NaOH) 255 nm (ε 24,300), 303 (20,400), 364 (9,220)] likewise suggested differences in electron availability at N^{10} depending on the position of the C=O group on the thiazole ring. In assays against *S. faecium*, (V.23) and (V.24) turned out to have some growth inhibitory activity (the pyridine analogues had supported growth). The IC_{50} value of the 4-substituted thiazole (V.23) was 6 μM, while that of the 5-substituted analogue (V.24) was 2.5 μM. There was thus a small increase in activity when the thiazole C=O group was at the 5-position where it could more effectively decrease electron availability at N^{10} through resonance. However, neither compound had anything like the potency of MTX, whose IC_{50} value against *S. faecium* was 3.5 nM. Although the precise mechanism by which (V.23) and (V.24) inhibited bacterial growth was not specified, it was noted that DHFR inhibition was unlikely to be the primary site of action, since the IC_{50} for inhibition of this enzyme required concentrations 30- to 60-fold higher than the IC_{50} for growth.



(V.23)

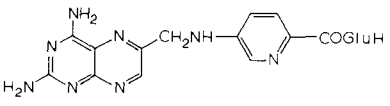


(V.24) X = N
(V.25) X = CH

In a further attempt to reduce the concept of N^{10} electron availability to practice, Roberts and Shealy [213] prepared 2′,6′-diazafolic acid (V.20) by an extension of the route used earlier to obtain (V.23) and (V.24). Comparison of the UV spectra of (V.20) [λ_{max} (0.1 M HCl) 245 nm (ε 13,400), 301 (22,900); λ_{max} (pH 7) 218 (ε 15,900), 230 (15,600), 283 (27,300); λ_{max} (0.1 M NaOH) 256 nm (ε 24,900), 288 (24,200), 362 (9,310)] and (V.19), especially at acid pH, suggested a greater effect on electron density at N^{10} in the 2′,6′-diaza than in the 2′-aza compound. Bioassay revealed that (V.20) was a poor DHFR inhibitor ($IC_{50} > 100$ μM) but, unlike (V.19), did inhibit *S. faecium* growth (IC_{50} = 10 nM) and had borderline activity (28% ILS at 100 mg/kg, qd × 9) against L1210 leukaemia in mice. It may be noted that a synthesis of the isomeric compound 2′,5′-diazafolic acid (V.21) was described independently by another group [214], though biological data were not provided. The 2′,3′-

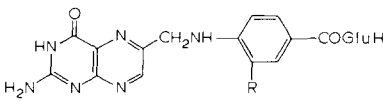
diaza analogue (V.22) has not been described. However, compound (V.25) was reported by Gurina *et al.* [210], who prepared it via a Waller synthesis from 2,4,5-triaminopyridin-6(1*H*)-one, 1,1,3-trichloroacetone, and *N*-(2-aminothiophene-5-carbonyl)-L-glutamic acid. This interesting bioisostere of folic acid was said to be a DHFR inhibitor and to block DNA synthesis in tumour cells, albeit only at high concentrations.

While folic acid analogues with a pyridine ring in Region D clearly had held no promise as antitumour agents, Montgomery *et al.* [14] did find activity in the interesting compound 2′-azaAMT (V.26), which they synthesized from diethyl *N*-(5-aminopicolinoyl)-L-glutamate by reaction with 2,4-diamino-6-bromomethylpteridine followed by alkaline hydrolysis. The IC_{50} value against pigeon liver DHFR was found to be 0.030 μM versus 0.025 μM for MTX, and the IC_{50} value for KB cell growth inhibition in culture was 0.014 μM versus 0.009 μM for MTX. High potency was also observed *in vivo* against L1210 leukaemia in mice, but there was also substantial dose-limiting toxicity; while the optimal therapeutic dose was a very low 0.44 mg/kg, the ILS at this dose was only 69%.



(V.26)

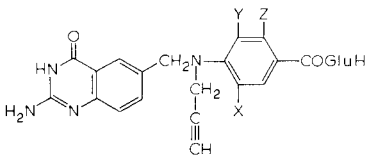
The effect of alkyl group substitution at the 3′-position of folic acid was investigated by Roberts and Shealy [216], who prepared the 3′-ethyl and 3′-isopropyl derivatives (V.27) and (V.28) by the route they had used to obtain other phenyl-ring-modified analogues. Neither compound was an inhibitor of DHFR from pigeon liver, and in fact the 3′-isopropyl derivative (V.28) appeared to *stimulate* reduction of dihydrofolate, leading the authors to propose that it was binding to a secondary site on the reductase. While (V.27) inhibited the growth of *S. faecium* with an IC_{50} value of 0.026 μM in the



(V.27) = Et
(V.28) = *i*-Pr
(V.29) = Me

presence of 25 nM folic acid, (V.28) was growth supportive at concentrations above 0.1 μM. Since activity was not observed in culture against KB human epithelial carcinoma cells or *in vivo* against L1210 leukaemia in mice, further work on these compounds was not pursued. It may be noted that the 3′-methyl derivative (V.29) of folic acid has been known since 1951 [217].

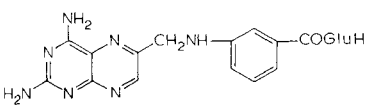
Phenyl ring substitutions were investigated by Jones *et al.* [218] as part of their intensive effort to develop TS inhibitors superior to N^{10}-propargyl-5,8-dideazafolic acid (CB3717) ((IV.287); *vide supra*). The 3′-chloro analogue (V.30) was obtained from the diethyl ester of (IV.287) in about 35% overall yield (two steps) by reaction with SO_2Cl_2 in $CHCl_3$ at room temperature followed by hydrolysis with NaOH in 50% EtOH. Only one Cl atom was introduced in this reaction, even though 2.5 molar equivalents of chlorinating agent were used. The 3′,5′-dichloro analogue (V.31) could be prepared, albeit in lower yield, by reaction of diethyl *N*-[4-(*N*-propargylamino)benzoyl]-L-glutamic acid with SO_2Cl_2 followed by coupling to 2-amino-6-bromomethylquinazolin-4(3*H*)-one (12% yield), and alkaline hydrolysis of the ester groups. The 2′-chloro and 2′-methyl analogues (V.32) and (V.33) were synthesized from the appropriate 2′-substituted diethyl *N*-(4-aminobenzoyl)-L-glutamates, which were prepared by routine procedures from 2-chloro- and 2-methyl-4-nitrobenzoyl chloride, respectively. The nitro group was reduced with sodium dithionite, rather than catalytically, when a chlorine atom was present. Alkylation of the 4-amino group was performed with propargyl bromide. Both propargylation and coupling to the bromomethylquinazoline proceeded in lower yield when the substituent *ortho* to the carbonyl group was Me rather than Cl, suggesting that the bulkier Me group may indirectly influence electron density on the nitrogen atom by causing partial loss of conjugation to the carbonyl group. This stereoelectronic effect is worth noting, since it may have considerable impact also on binding to TS. That electron density at C-9 and propargyl CH_2 group could in fact be influenced by the



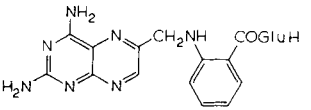
(V.30) X = Cl, Y = Z = H
(V.31) X = Y = Cl, Z = H
(V.32) X = Y = H, Z = Cl
(V.33) X = Y = H, Z = Me

phenyl ring substituent was clear from NMR spectra. For example, while the protons on these groups gave rise to singlets at δ 4.45 and δ 3.90, respectively, in (V.31) (3′,5′-dichloro), the signals for the corresponding protons in (V.33) (2′-methyl) were at δ 4.6 and δ 4.2. The lower-field location of the resonances for (V.31) are consistent with decreased electron density at N^{10}, and hence diminished shielding of nearby hydrogens.

The question of *para* versus *meta* or *ortho* substitution on the phenyl ring has been examined recently [219]. The *N*-(3-nitrobenzoyl) and *N*-(2-nitrobenzoyl) derivatives of diethyl-L-glutamate were reduced catalytically, the resultant amines were condensed with 2,4-diamino-6-bromomethylpteridine, and the ester groups were cleaved with base to obtain '*meta*AMT' (V.34) and '*ortho*AMT' (V.35), respectively. Yields were low but enough material was obtained for limited *in vitro* testing. In assays against DHFR from L1210 cells, (V.34) had an IC_{50} value of 200 μM as compared with 0.002 μM for AMT; (V.35) was similarly inactive (20% inhibition at 50 μM). Growth inhibitory activity against L1210 cells in culture was likewise poor, the IC_{50} of (V.34) being 1.5 μM as compared with 0.002 μM for AMT, while that of (V.35) rose to 64 μM. While the cytotoxicity of these compounds was obviously discouraging, an unusual aspect of the results was that the IC_{50} of (V.34) against cultured cells was about 100-fold lower than its IC_{50} value as a DHFR inhibitor, suggesting that inhibition of cell growth might be due to action at a biochemical locus other than DHFR. It is of interest that the *meta* isomer of folic acid has been reported to have some antifolate activity in culture [220].



(V.34)

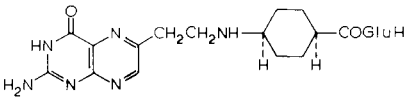


(V.35)

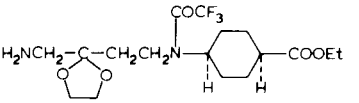
Two additional modifications of Region D deserve to be mentioned, one being the homofolic acid analogue (V.36) (phenyl ring replaced by cyclohexane ring), and the second being the MTX analogue (V.38) (phenyl ring replaced by open tetramethylene chain).

1′,2′,3′,4′,5′,6′-Hexahydrohomofolic acid (V.36) was synthesized by Nair *et al.* [221] by condensation of 2,4-diamino-6-chloro-5-nitropyrimidine with the amino ketal (V.37), followed by ketal acidolysis, reductive cyclization to a 7,8-dihydropteridine, aerobic oxidation, ester hydrolysis, N^2,N^{11}-bis(trifluoroacetylation), mixed anhydride condensation with diethyl L-glutamate, and cleavage of the ester and amide groups with base. Ketal (V.37) was

obtained by straightforward elaboration of *N*-(4-ethoxycarbonylphenyl)-*β*-alanine. Although the phenyl ring was reduced catalytically, which would be expected to yield a 1,4-*cis* product, the possibility of epimerization to the more stable 1,4-*trans* isomer at a subsequent stage of the synthesis was not ruled out. The amino ketal system was generated via a modified Arndt-Eistert sequence, after N-protection with trifluoroacetic anhydride. Compound (V.36) was inactive as either a substrate or inhibitor of DHFR from *L. casei*, and its tetrahydro derivative was not a pseudocofactor for TS from the same organism. Similarly, growth inhibition was not observed against *S. faecium*, *L. casei*, or KB cells in culture. Replacement of the planar phenyl ring in homofolic acid by a chair-like cyclohexane ring was clearly detrimental to biological activity, though the reasons were not specified.

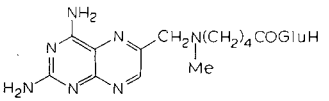


(V.36)



(V.37)

Montgomery *et al.* [14] synthesized the 2,4-diaminopteridine derivative (V.38) by condensation of 2,4-diamino-6-bromomethylpteridine with diethyl *N*-(5-*N*-methylaminopentanoyl)-L-glutamate followed by alkaline hydrolysis. The binding affinity of (V.38) for pigeon liver DHFR (IC_{50} = 20 μM) was 740-fold lower than that of MTX (IC_{50} = 0.027 μM), and no significant activity against KB cells in culture or L1210 leukaemia in mice was observed. Replacement of the planar phenyl ring by a simple hydrophobic alkyl moiety was therefore not tolerated any better in MTX than in homofolic acid.



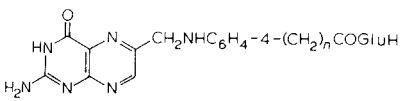
(V.38)

CHANGES IN REGION E

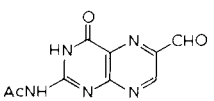
As defined in the Introduction, Region E consists of the amide and α-carbon moieties jointly contributed by the *p*-aminobenzoyl and glutamate portions of the folate structure. Modifications of this region have been few and relatively

unproductive in terms of biologically active compounds, but are nonetheless important to consider in the total context of antifolate structure–activity relationships.

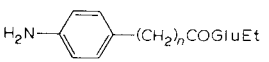
The earliest attempt to study folate analogues modified in Region E appears to have been that of Roberts and Shealy [222], who synthesized 'neohomofolic acid' (VI.1) and 'neobishomofolic acid' (VI.2) with the aim of insulating N^{10} from the electron-withdrawing effect of the CONH group. The underlying hypothesis for this effort was that these analogues might be reduced by DHFR in the cell and then converted to fraudulent 'one-carbon donors' (for example, 5,10-methylene derivatives). If decreased electron density at N^{10} prevented one-carbon transfer while still allowing binding to an enzyme (for example, thymidylate synthase), one might expect competitive inhibition. 2-Acetamido-4(3*H*)-oxopteridine-6-carboxaldehyde (VI.3) was allowed to react with diethyl *N*-(4-aminophenylacetyl)-L-glutamate (VI.4) in DMSO to obtain a Schiff's base, which was sequentially reduced with $NaBH_4$ in DMF and hydrolyzed anaerobically with 0.2 M NaOH to obtain (VI.1) in 29% purified yield. At a molar ratio of $NaBH_4$ to Schiff's base of 1.25:1.00, reduction of the pyrazine ring was found to be negligible. A similar sequence starting from diethyl *N*-(4-aminobenzoyl)-L-glutamate afforded folic acid (48%), while diethyl *N*-[3-(4-aminophenyl)propionyl]-L-glutamate (VI.5) gave (VI.2) (14%). The amino esters (VI.4) and (VI.5) were obtained from diethyl L-glutamate and 4-nitrophenylacetic and 4-nitrocinnamic acids, respectively, by carbodiimide coupling followed by catalytic reduction (H_2-RaNi). As expected from the 'decoupling' of the N^{10} and CONH moieties, the UV spectra of (VI.1) [λ_{max} (0.1 M HCl) 232 nm (ε 15,400), 245 (13,900), 282 (4,100); λ_{max} (pH 7) 242 nm (ε 22,700), 272 (17,600), 346 (7,100); λ_{max} (0.1 M NaOH) 254 nm (ε 33,900), 275 sh, 365 (8,700)] differed markedly from those of folic acid [λ_{max} (0.1 M HCl) 246 nm (ε 13,200), 296 (19,400); λ_{max} (pH 7) 281 (ε 27,400), 346 (7,100); λ_{max} (0.1 M NaOH) 256 (ε 24,300), 283 (23,900), 364 (8,500)] and were nearly identical to those of (VI.2). Unfortunately, biological activity results were disappointing. Inhibition of pigeon liver DHFR occurred only at concentrations exceeding 100 μM (versus 0.01 μM for MTX), the IC_{50} value against *S. faecium* was > 10 μM (versus 0.0007 μM for MTX), and there was no



(VI.1) n = 1
(VI.2) n = 2



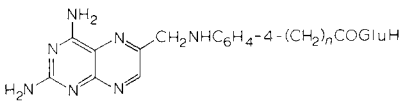
(VI.3)



(VI.4) n = 1
(VI.5) n = 2

significant inhibition of either KB cell growth in culture or the progression of L1210 leukaemia in mice. Since neither (VI.1) nor (VI.2) supported the growth of *S. faecium*, it was clear that structural changes in Region E of folic acid brought about a major, albeit unspecified, change with respect to one or more of the enzymes in the folate pathway of this organism.

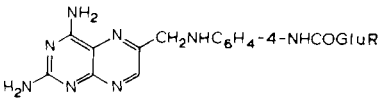
In order to determine what effect insertion of a CH_2 spacer between the phenyl ring and CONH moiety would have on DHFR binding, Montgomery *et al.* [14] synthesized the AMT analogues (VI.6) (72% yield) and (VI.7) (38% yield) by condensing with *N*-(4-aminophenylacetyl)- and *N*-[3-(4-aminophenyl)propionyl)-L-glutamic acid, respectively, with a 3:1 excess of 2,4-diamino-6-bromomethylpteridine in DMA. The acids were prepared from the corresponding diesters by alkaline hydrolysis. The inhibitory activity of (VI.6) against pigeon liver DHFR was 15-fold lower than that of MTX, while that of (VI.7) was 38-fold lower. The IC_{50} value of both compounds against KB human epithelial carcinoma cells in culture was in the 5–10 μM range, and was 10^4-fold higher than that of MTX. Against L1210 leukaemia in mice, (VI.6) produced a marginal 35% ILS at the maximally tolerated dose of 500 mg/kg (qd × 9), while (VI.7) was inactive; the optimal dose of MTX on the same schedule was 0.67 mg/kg. It was clear from these results that insertion of a CH_2 spacer between the phenyl and CONH moiety led to a decrease in DHFR affinity, but was probably even more detrimental to transport (and perhaps polyglutamylation). The authors did not speculate as to the reasons for the loss of activity associated with this structural alteration. However, possible consequences of CH_2 insertion might be that the α-COOH group becomes less acidic, or that it is less favourably positioned for ionic interaction with a basic amino-acid residue in the enzyme active site.



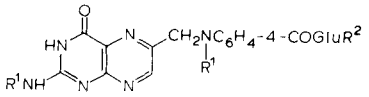
(VI.6) $n = 1$
(VI.7) $n = 2$

A different type of modification in Region E was achieved [223, 224] in the form of the MTX analogue (VI.8) and the folic acid analogue (VI.10), in which NH is inserted between the phenyl and CONH moiety. For the synthesis of (VI.8), 4-amino-4-deoxy-N^{10}-methylpteroic acid was treated with diphenylphosphoryl azide in DMSO, the resultant azide ((VI.12), 87% yield) was photolyzed in DMF solution in the presence of dimethyl L-glutamate to obtain dimethyl ester (VI.9) (51% yield), and (VI.9) was hydrolyzed with 0.1 M

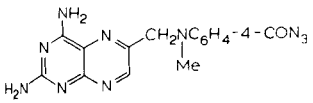
NaOH in MeOH (1.5 h at room temperature, 79% yield). For the synthesis of (VI.10), N^2,N^{10}-diacetylpteroic acid was converted to the azide (VI.13) (83% yield) with diphenylphosphoryl azide in DMF, (VI.12) was heated in refluxing xylene for 25 min, the resulting isocyanate (not isolated) was treated with dimethyl L-glutamate to form diester (VI.11) (40% yield). Unfortunately, while cleavage of the esters was rapid, hydrolysis of the amide groups in (VI.11) required vigorous conditions (2 M NaOH, 3 h reflux) that led to extensive decomposition and produced (VI.10) in only 10% yield. Photocatalyzed coupling was not satisfactory in the case of (VI.11) because normal peptide coupling competed with Curtius rearrangement, giving dimethyl N^2,N^{10}-diacetylfolate as a major byproduct. In an alternative route to (VI.10), 4-aminobenzoyl azide was photolyzed in the presence of dimethyl L-glutamate, the resultant urea ((VI.14), 30% yield) was allowed to form a Schiff's base with 2-acetamido-4(3*H*)-oxopteridine-6-carboxaldehyde in acetic acid, the C^9–N^{10} double bond was reduced directly with dimethylamine borane (66% yield), and the amide and ester groups were hydrolyzed with 0.1 M NaOH in MeOH (5 h at room temperature, 60% yield).



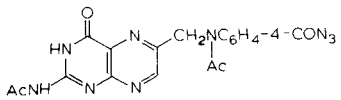
(VI.8) R = H
(VI.9) R = Me



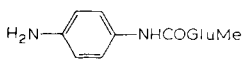
(VI.10) $R^1 = R^2 = H$
(VI.11) R^1 = Ac, R^2 = Me



(VI.12)



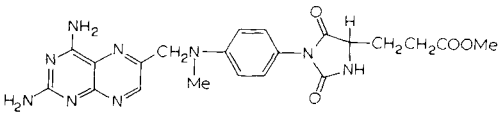
(VI.13)



(VI.14)

An unexpected feature of the chemistry of the above ureido ester analogues was the apparent ease with which they underwent at least partial racemization at the α-carbon during alkaline hydrolysis to ureido acids. Support for the conclusion that racemization was taking place came from a combination of direct polarometric measurements on the ureido diacids (low rotation in comparison with MTX and folic acid) and indirect evidence that hydrolysis was proceeding via a hydantoin intermediate. Thus, when the ester (VI.9) was heated in MeOH or $CHCl_3$ in the presence of the strong organic base 1,5-diazabicyclo[3.4.0]non-5-ene, a product formed in 61% yield which was tentatively identified as (VI.15). Alkaline hydrolysis of (VI.15) yielded (VI.8).

The authors argued that since (i) a hydantoin formed readily from (VI.9) in the presence of base and (ii) chiral *N*-arylhydantoins are known to racemize easily in base, (VI.9) most probably cyclized to hydantoin (VI.15) prior to ester cleavage. It should be noted that polarometric data for (VI.8) and (VI.10) were obtained only at the D line of sodium, and that the reliability of such data may be limited because pteridines absorb light very strongly at this wavelength. Furthermore, the finding that (VI.8) and (VI.10) had a rotation of less than one degree did not, in and of itself, mean that the compounds were racemic. Thus, more rigorous evidence is probably needed in order to settle this issue.

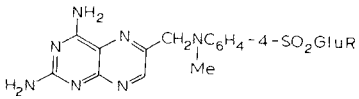


(VI.15)

In assays against DHFR from *L. casei*, the diester (VI.9) and diacid (VI.8) were found to have IC_{50} values of 0.08 and 0.3 μM, respectively, as compared with 0.03 μM for MTX. Thus, introduction of an extra NH between the phenyl and CONH moiety caused only a 2- to 3-fold decrease in DHFR affinity (which could be due mainly to the fact that the compound was racemic), while esterification led to an additional 4-fold decrease in binding. When (IV.8) and (VI.9) were tested as inhibitors of intact *L. casei*, on the other hand, they were found to have IC_{50} values of 0.016 and 0.018 μM as compared with 0.022 nM for MTX. The 1000-fold greater sensitivity of *L. casei* to MTX than to (VI.8) was in marked contrast to the comparable DHFR affinities of the two drugs, and suggested that the 'aza homologue' was not taken up well by the organism and/or did not form polyglutamates. Not surprisingly, the hydantoin (VI.15) was a relatively poor DHFR inhibitor (IC_{50} = 4 μM) and its activity against *L. casei* was approx. 10-fold lower than that of (VI.8). In assays against CEM cells in culture, the acid (VI.8) had an IC_{50} value of 0.8 μM while the ester (VI.9) had an IC_{50} value of 6.3 μM and was thus 8-fold less potent. When (VI.8) was administered to mice with L1210 leukaemia at a dose of 200 mg/kg (q4d × 3), it produced a 55% ILS. Treatment with 30 mg/kg of MTX on the same schedule gave an 88% ILS. Thus, the optimal therapeutic dose of VI.8 was roughly 10-fold higher than that of MTX, in good agreement with its lower *in vitro* potency.

The folic acid analogue (VI.10) was a weak inhibitor of *L. casei* TS (IC_{50} = 180 μM) and was inactive against *L. casei* (IC_{50} > 4 μM) and CEM cells (IC_{50} > 20 μM). In addition, (VI.10) supported the growth of *S. faecium*,

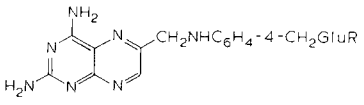
and its tetrahydro derivative supported the growth of *P. cerevisiae*; however, neither compound was as effective as the natural growth factors folic and folinic acid. It is conceivable that some of this observed growth-supporting effect was due to the presence of trace amounts of folic acid in the test sample. No *in vivo* activity against L1210 leukaemia was observed at the highest tolerated dose (60 mg/kg, q4d × 3). The reason for the greater toxicity of (VI.10) in comparison with MTX is unclear.



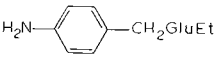
(VI.16) R = Et
(VI.17) R = H

Replacement of the CO group in the amide function of MTX by SO_2 was accomplished by Montgomery *et al.* [14]. Condensation of diethyl *N*-(4-aminobenzenesulphonyl)-L-glutamate with 2,4-diamino-6-bromomethylpteridine gave the diester (VI.16), which on alkaline hydrolysis yielded the diacid (VI.17). The inhibitory activity of (VI.17) against pigeon liver DHFR (IC_{50} = 1.1 μM) was 40-fold lower than that of MTX, its growth inhibitory potency against KB cells (IC_{50} = 100 μM) was 10^4-fold lower, and it displayed only marginal activity against L1210 leukaemia in mice at a dose of 100 mg/kg (qd × 9). Thus replacement of the CONH in MTX by SO_2NH is very detrimental to biological activity, possibly because the CONH oxygen can serve as a hydrogen-bond acceptor in the enzyme-inhibitor complex, a role that cannot be filled by the SO_2NH oxygen.

A further example attesting to the importance of the amide CO was provided by Rosowsky and Forsch [225] in the form of 'deoxoaminopterin' (VI.18), in which the CONH moiety was replaced by CH_2NH. Diethyl L-glutamate was condensed with 4-nitrobenzaldehyde, the resulting Schiff's base was reduced catalytically to amine (VI.20), the product was condensed with 2,4-diamino-6-bromomethylpteridine in DMA to obtain the diester (VI.19), and the ester groups were removed by hydrolysis with $Ba(OH)_2$. Since the amine (VI.20) contained two potential sites of alkylation, it was necessary to prove that 2,4-diamino-6-bromomethylpteridine had reacted with the aniline nitrogen. This was done by demonstrating that both (VI.19) and (VI.18) lacked a diazotizable NH_2 group as determined by a Bratton-Marshall reaction, whereas amine (VI.20) gave the expected positive test. In a spectrophotometric assay using DHFR from *L. casei*, VI.18



(VI.18) R = H
(VI.19) R = Et

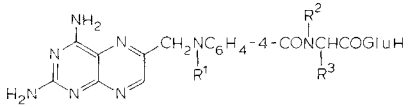


(VI.20)

(IC_{50} = 0.47 μM) was found to be 17-fold less inhibitory than AMT (IC_{50} = 0.027 μM), while in a competitive binding assay using lysates from L1210 murine leukaemia cells as the source of enzyme and [^{3}H]MTX as the radioligand the IC_{50} values obtained with (VI.18) and AMT were 0.024 and 0.0022 μM, respectively. There was thus a 10- to 20-fold loss of binding affinity for both bacterial and mammalian DHFR upon replacement of CONH by CH_2NH. In cell culture against L1210 cells, (IV.18) was devoid of growth inhibitory activity even at 1 μM, while *in vivo* against L1210 leukaemia in mice, no increase in lifespan was observed at the highest nontoxic dose tested (240 mg/kg, q3d × 3). It thus appeared that (VI.18) was even less active than AMT as an antitumour agent than as a DHFR inhibitor, indicating that decreased DHFR binding was probably not the sole reason for the loss of activity associated with replacement of the amide CO by CH_2. Possible additional reasons for the inactivity of (VI.18) might be inefficient cellular uptake. Alkylation, as opposed to acylation, of the α-amino group in glutamic acid should bring about an increase in the 'average pK_a' for the two COOH groups of approximately 1 unit, corresponding to a 10-fold decrease in concentration of the non-ionized form at physiologic pH. This could have a negative effect on the cellular uptake of (VI.18) in comparison with AMT if it is true, as has been suggested [226, 227], that the species actually penetrating the cell membrane is the undissociated (that is, non-charged) one, even though it constitutes only a small fraction of total extracellular drug. A decrease in pK_a for the α-COOH group might also be detrimental to DHFR binding. Decreased DHFR binding likewise might be expected if, as suggested by X-ray crystallographic studies [228–230], hydrogen bonding of the amide C=O group to an amino acid in the active site plays a part in stabilizing the enzyme-inhibitor complex. Finally, Moran *et al.* [194] have observed that (VI.18) is a very poor substrate for folylpolyglutamate synthetase, which would be expected to also have a detrimental effect on biological activity, especially *in vivo*.

Suster *et al.* [231] have described several AMT and MTX analogues with hydrophobic amino acid 'spacers' between the pteroyl and glutamate moieties. Condensation of ethyl glycinate with 4-(*N*-Cbz-amino)benzoyl chloride, followed by alkaline hydrolysis of the ester group, DCC coupling to diethyl

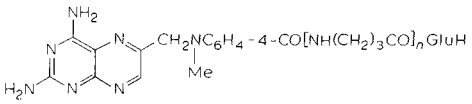
L-glutamate, another alkaline hydrolysis, and finally hydrogenolysis of the Cbz group and coupling to 2,4-diamino-6-chloromethylpteridine afforded the AMT analogue (VI.21). The identical sequence starting from 4-(*N*-Cbz-*N*-methylamino)benzoyl chloride yielded the MTX analogue (VI.22). Likewise prepared by this general approach, in overall yields of 8–12%, were the MTX analogues (VI.23)–(VI.26), containing DL-alanine, sarcosine, L-leucine and L-phenylalanine, respectively, as the 'spacer'. As should perhaps have been anticipated, none of these compounds showed antitumour activity, presumably because of negative characteristics such as low DHFR affinity, slow uptake, and lack of conversion to polyglutamates. It was of interest, however, that two members of the series in which the L-glutamic acid moiety at the end of the side-chain was replaced by L-aspartic acid did show some *in vivo* antitumour activity, as described in the next section.



(VI.21) $R^1 = R^2 = R^3 = H$
(VI.22) $R^1 = Me$, $R^2 = R^3 = H$
(VI.23) $R^1 = R^3 = Me$, $R^2 = H$
(VI.24) $R^1 = R^2 = Me$, $R^3 = H$
(VI.25) $R^1 = Me$, $R^2 = H$, $R^3 = CH_2CH(CH_3)_2$
(VI.26) $R^1 = Me$, $R^2 = H$, $R^3 = CH_2C_6H_5$

A different type of modification of Region E has involved the introduction of up to five 4-aminobutyryl (Gab) 'spacers' between the *p*-aminobenzoyl and L-glutamate moieties of MTX [232, 233]. The prototype of this series, *N*-[*N*-(4-amino-4-deoxy-N^{10}-methylpteroyl)amino]butyric acid (VI.27), was obtained in 92% yield from 4-amino-4-deoxy-N^{10}-methylpteroic acid by reaction with the *O*,*N*-bis(trimethylsilyl) derivative of 4-aminobutyric acid in the presence of diethyl phosphorocyanidate as the coupling reagent. The homologous 'stretched' MTX analogues (VI.28)–(VI.31) were obtained from the corresponding di-*t*-butyl esters by acid hydrolysis at room temperature (TFA, 10 min). The esters were synthesized in 25–50% yield from 4-amino-N^{10}-methylpteroic acid and the peptides $H_2N(CH_2)_3CO[NH(CH_2)_3CO]_nNH$-$CH(COOBu\text{-}t)CH_2CH_2COOBu\text{-}t$ ($n = 1$–4). The latter formed readily from di-*t*-butyl L-glutamate and the acids $CbzNH(CH_2)_3CO[NH(CH_2)_3$-$CO]_nOH$ ($n = 1$–4) on mixed anhydride coupling followed by catalytic hydrogenolysis of the Cbz group. Compounds (VI.28)–(VI.31) may be viewed as

analogues of the MTX oligoglutamates with all the α-carboxyl groups deleted except the one at the end of the side-chain.



(VI.27)—(VI.31) $n = 1—5$

Assays of DHFR inhibition by VI.27–VI.31 were carried out with purified enzyme from L1210 cells in order to determine whether Gab 'spacers' are as well tolerated as are the several glutamyl residues in MTX oligoglutamates [233]. There was only a 2- to 3-fold difference in IC_{50} value between MTX and the analogues (VI.27) and (VI.28), which contained one and two Gab spacers, respectively. With the introduction of a third Gab (VI.29) the difference in IC_{50} value increased to 10-fold, and by the time five Gab units had been added (VI.31) the difference increased to 24-fold. These results confirmed that the α-COOH group on the glutamate residue nearest to the *p*-aminobenzoyl group is important for tight binding to DHFR, and moreover supported the idea that 'internal' α-COOH groups in MTX oligoglutamates likewise contribute to binding.

Since other workers [182, 183, 234, 235] had shown that MTX polyglutamates were better inhibitors of TS than is MTX itself, it was of interest to determine whether a similar trend existed among compounds (VI.27)–(VI.31). In assays against purified TS from *L. casei,* the K_i values of the 'stretched' MTX analogues with Gab spacers were found to be in the 0.1–0.4 μM range, comparing favorably with the K_i values of MTX polyglutamates of the same chain length, which are reported to be in the 0.05–0.2 μM range against *L. casei* enzyme [234]. Binding increased as the number of Gab spacers went from one to three, but did not increase thereafter. Overall, the K_i values of (VI.27)–(VI.31) as inhibitors of *L. casei* TS approximated those of MTX polyglutamates and were substantially lower than the K_i of MTX itself. Taken together with the DHFR data, the results suggested that 'stretched' MTX analogues such as (VI.27)–(VI.31) might have the potential to kill cells by combined inhibition of both enzymes. When these compounds were subsequently tested as inhibitors of TS in *permeabilized* L1210 murine leukaemia cells, however, they proved to have less activity than had been seen *in vitro* against bacterial enzyme. This suggested either that the binding specificity of the mammalian enzyme was different from that of the bacterial enzyme, or that the compounds were being metabolized in the cells to products with

decreased ability to bind to TS. In growth inhibition assays with intact L1210 cells, (VI.27), (VI.28) and (VI.29) were found to have IC_{50} values of 0.53, 5.6 and 29 μM, while (VI.30) and (VI.31) were inactive below 100 μM.

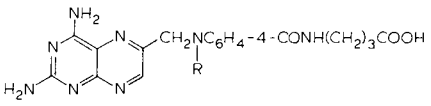
An interesting observation made with compound (VI.27) was that this molecule was accepted as a substrate by FPGS in a cell-free assay, and thus was likely to undergo at least some polyglutamylation in intact cells [232]. The V_{max} for glutamate addition to (VI.27) at a saturating concentration was estimated to be about the same as the V_{max} for MTX + G_1, although the concentration for half-saturation was substantially higher. The longer-chain congeners (VI.28)–(VI.31), on the other hand, were essentially devoid of FPGS substrate activity. Apparently, (VI.27) does not bind as tightly as MTX + G_1 to the enzyme, but once it binds (and does so in the correct configuration), addition of at least one glutamyl residue to the terminal COOH is possible. Moreover the results of this study suggested that reaction catalyzed by FPGS is possible when the α-COOH group of the terminal glutamate is relatively close to the pteroyl moiety, as in (VI.27), but not when the pteroyl moiety and α-COOH group are separated by more than the length of one Gab spacer.

CHANGES IN REGION F

An acidic group of the α-carbon of the glutamate side-chain in classical antifolates appears to be essential for potent *in vitro* and *in vivo* biological activity, as evidenced by the studies to be summarized below. Three types of structural modification have been made in this region of the molecule: (i) outright deletion of the α-carboxyl group, (ii) replacement of the α-carboxyl group by another acidic group, and (iii) blocking of the α-carboxyl group in the form of an ester, amide, or peptide derivative.

Several MTX analogues in which the glutamate side-chain was replaced by other amino acids were reported in 1978 by Suster *et al.* [231]. Among the compounds synthesized by these authors was *N*-(4-amino-4-deoxy-N^{10}-methylpteroyl)-4-aminobutyric acid (VII.1), which differed from MTX only in lacking a carboxyl group at the α-position. Acylation of ethyl 4-aminobutyrate with 4-(*N*-Cbz-*N*-methylamino)benzoyl chloride, followed by alkaline hydrolysis of the ester group and hydrogenolysis of the Cbz group, gave *N*-[4-(*N*-methylamino)benzoyl]-4-aminobutyric acid. Condensation with 2,4-diamino-6-chloromethylpteridine, which was obtained from 2,4-diamino-6-hydroxymethylpteridine and PCl_3, then gave (VII.1). The overall yield was < 10%, but enough material was obtained for *in vivo* assays against L1210

leukaemia in mice and Walker 256 carcinoma in rats. No increase in survival at all was noted against L1210 leukaemia at a dose of 10 mg/kg (qd × 8). Against the rat carcinoma, however, which is generally regarded as being more responsive than L1210 leukaemia to certain types of antifolate, especially those with lipophilic character, a 55% ILS was observed at a dose of 8 mg/kg (qd × 14). These results were interesting in that they established beyond doubt that the α-COOH group is absolutely essential for activity against L1210 leukaemia, but also suggested that there might be other tumours for which this requirement may be less critical. The latter point has perhaps not received the attention it deserves. Studies of the effect of (VII.1) on DHFR activity and the growth of cells in culture have not been reported, but it is reasonable to assume on the basis of work performed with the corresponding AMT analogue (*vide infra*) that activity would be substantially decreased in comparison with MTX. A point worth noting is the finding of Moran *et al.* [232] that (VII.1) is an extremely poor substrate for folylpolyglutamate synthetase (FPGS). Thus, even if (VII.1) were a good DHFR inhibitor, its therapeutic dose would be expected to be very high in comparison with MTX, which is efficiently converted to non-effluxing polyglutamates. When assayed as a competitive inhibitor of MTX polyglutamylation by FPGS, (VII.1) was found inactive, indicating that deletion of the α-COOH group markedly decreases binding to this enzyme, just as it decreases binding to DHFR and TS.

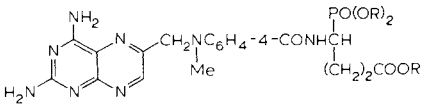


(VII.1) R = Me
(VII.2) R = H

The AMT analogue VII.2, similarly lacking a carboxyl group at the α-position, was prepared by Montgomery *et al.* [14] in 63% overall yield by condensation of ethyl 4-aminobutyrate with 2,4-diamino-6-bromomethylpteridine followed by alkaline ester hydrolysis. When (VII.2) was tested as an inhibitor of DHFR from pigeon liver, it was found to have an IC_{50} value of 0.38 μM, corresponding to a 15-fold loss of potency relative to MTX in the same experiment. Although comparison with AMT would have been more appropriate, these results pointed to the α-COOH group as contributing significantly to binding to the avian enzyme. In assays against KB human epithelial carcinoma cells in culture, (VII.2) was inactive below 0.24 μM and thus was at least 40-fold less potent than MTX. Against L1210 leukaemia in

mice, no increase in survival was observed at doses of up to 25 mg/kg (qd × 9), a finding consistent with that of Suster *et al.* [231] with (VII.1).

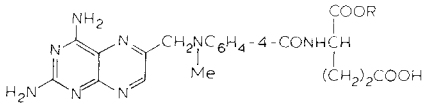
The question of whether replacement of the α-COOH group in MTX by another acidic group such as $PO(OH)_2$ or SO_2OH remains open, but may be answered in the near future. In a recent paper, Sturtz and Guillamot [236] somewhat sketchily described the synthesis of the triethyl ester (VII.3), which on hydrolysis would be expected to give the acid (VII.4). The synthesis of (VII.3) was accomplished by coupling triethyl *N*-[4(*N*-methylamino)benzoyl]-2-amino-2-phosphonobutanoate to 2-amino-5-bromomethylpyrazine-3-carbonitrile and condensing the product with guanidine. The amino diester was obtained from 4-(*N*-Cbz-*N*-methylamino)benzoyl chloride and triethyl 2-amino-2-phosphonobutanoate. Cleavage of the ester groups in (VII.3) was not described, but ought to be possible on treatment with Me_3SiBr followed by MeOH as in the reaction of an analogous α,γ-diphosphonic acid derivative also synthesized by these authors [236] (*vide infra*).



(VII.3) R = Et
(VII.4) R = H

α-Monoesters of MTX containing one to four alkyl carbons were synthesized in 1978 by Rosowsky *et al.* [237] by acid-catalyzed esterification of MTX in the presence of a limited amount of HCl or by partial saponification of the α,γ-diesters with NaOH. Generally the α-esters were formed in smaller amount than the isomeric γ-esters, and were accompanied by the diesters as well as by MTX. Separation of α- and γ-monoesters was found to be possible on silica-gel columns, with the more acidic γ-esters always eluting more slowly than the α-esters. Separation of α- and γ-esters by preparative HPLC on reversed phase C_{18} silica gel was also possible [238]. α-Monoesters obtained in low yield (< 15%) in this manner included the ethyl (VII.5) and *n*-butyl (VII.6). More recently, it was found [239] that mixtures of α-monoesters (5–10% yield), γ-monoesters (10–15% yield), and diesters (20–40% yield) could also be obtained from MTX by reaction with an alkyl bromide (1 equiv.) in the presence of Cs_2CO_3 (1 equiv.) in DMSO at room temperature. Structure assignments for the α-monoesters were based as before on chromatographic behaviour, and also on the fact that NMR spectra of the α-alkyl γ-2,6-dichlorobenzyl esters were different from those of γ-alkyl α-2,6-dichlorobenzyl esters because of the greater deshielding effect of the α-carboxyl

C=O group. α-Monoesters of MTX prepared by the Cs_2CO_3 method included the *n*-octyl (VII.7), *n*-dodecyl (VII.8), and *n*-hexadecyl (VII.9).



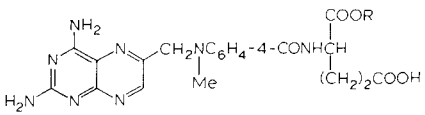
(VII.5) R = Et
(VII.6) R = *n*-Bu
(VII.7) R = *n*-C_8H_{17}
(VII.8) R = *n*-$C_{12}H_{25}$
(VII.9) R = *n*-$C_{16}H_{33}$

In spectrophotometric assays of beef liver DHFR inhibition, (VII.6)–(VII.9) gave IC_{50} values of 0.056, 0.36, 0.44 and 1.2 μM, respectively, while MTX had an IC_{50} value of 0.0033 μM [239]. These results clearly demonstrated (i) that esterification of the α-carboxyl group decreased DHFR binding, and (ii) that the binding of α-monoesters followed the order *n*-butyl > *n*-dodecyl > *n*-hexadecyl (*i.e.*, that hydrophobic substitution in the α-region was detrimental to enzyme binding). Each α-monoester was also found to be a poorer inhibitor than the corresponding γ-monoester.

The ability of α-monoesters to inhibit the growth of cultured cells was evaluated against CEM human leukaemic lymphoblasts [237, 239]. The IC_{50} values of esters (VII.5)–(VII.8) were determined to be in the 2 to 6 μM range, as compared with 0.025 μM for MTX. Surprisingly, the IC_{50} value of (VII.9) was found to be 0.25 μM. Thus, the α-*n*-hexadecyl ester was only 10-fold less growth-inhibitory than MTX and its activity relative to α-monoesters of shorter chain length was greater than would be predicted on the basis of DHFR binding data. It was suggested that the lipophilic *n*-hexadecyl group, in addition to promoting passive diffusion into the cell, could also lead to the formation of micellar aggregates capable of being taken up by endocytosis. Since the ester also had the potential to be cleaved enzymatically inside the cell, thereby acting as a prodrug of MTX, the relatively high activity of (VII.9) was perhaps an indication that the α-*n*-hexadecyl ester group was a particularly good substrate for cellular esterases.

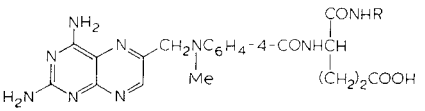
General synthetic methods of preparation of α-monoesters and α-monoamides of MTX were also described by Piper *et al.* [240]. The key intermediate in their scheme was 2,4-diamino-6-bromomethylpteridine, which could be condensed, for example, with α-methyl *N*-[4-(*N*-methylamino)benzoyl]-L-glutamate or *N*-[4-(*N*-methylamino)benzoyl]-L-glutamic acid α-amide

to form the α-methyl ester (VII.10) (89% yield) or α-amide (VII.11) (96% yield), respectively.



(VII.10) R = OMe
(VII.11) R = NH_2

MTX analogues (VII.12)–(IV.21), in which the α-carboxyl group is blocked as an alkylamide, were reported in 1984 by Antonjuk *et al.* [241]. The rationale for the synthesis of these compounds was that they might serve as lipophilic prodrugs. The α-amide (VII.11) was also prepared. Activation of *N*-Cbz-L-glutamic acid as a mixed anhydride (iBuOCOCl-Et_3N) followed by addition of a primary amine gave a mixture of α- and γ-amides, which on treatment with *t*-BuOAc and 70% $HClO_4$ was converted to a mixture of α-amide γ-*t*-butyl esters and γ-amide α-*t*-butyl esters. The α-amide γ-*t*-butyl esters, which were predominant in most instances and were unequivocally identified by NMR analysis, were separated by chromatography, deprotected by catalytic hydrogenation, and allowed to react with 4-(*N*-methyl-*N*-trifluoroacetyl)aminobenzoyl chloride. The resultant *N*-[4-(*N*-methyl-*N*-trifluoroacetyl)aminobenzoyl]-L-glutamic acid α-amide γ-*t*-butyl esters were then condensed with 2,4-diamino-6-bromomethylpteridine. Acidolysis of the *t*-butyl esters with anhydrous TFA afforded the desired α-monoalkylamides (VII.12)–(IV.21). The α-amide (VII.11) was prepared by condensation of *N*-[4-(*N*-methyl-*N*-trifluoroacetyl)aminobenzoyl]-L-glutamic acid α-amide γ-*t*-butyl ester with 2-amino-5-bromomethylpyrazine-3-carbonitrile *N*-oxide, followed by deoxygenation with $(EtO)_3P$ in DMF at 145–150 °C, annulation with guanidine in boiling *t*-BuOH, and acidolysis of the *t*-butyl ester with anhydrous TFA.



(VII.12) R = Me
(VII.13) R = Et
(VII.14) R = *i*-Pr
(VII.15) R = *n*-Pr
(VII.16) R = *t*-Bu
(VII.17) R = *sec*-Bu
(VII.18) R = *i*-Bu
(VII.19) R = *n*-Bu
(VII.20) R = cyclohexyl
(VII.21) R = $PhCH_2$

The effect of α-amidation on DHFR binding and cell growth inhibition has been evaluated [240]. The K_i of (VII.11) as an inhibitor of DHFR from L1210 cells was 10^5-fold greater than that of MTX, attesting to the importance of the free α-COOH group for binding. The IC_{50} of (VII.11) against cultured H.ep.2 cells was 0.61 μM, whereas that of MTX was 0.0024 μM. Thus, α-amidation resulted in a 250-fold loss of potency in the cell culture assay which was much smaller than the differential in DHFR binding, possibly reflecting intracellular cleavage of (VII.11) to free MTX by amidases. *In vivo* against L1210 leukaemia in mice, (VII.11) at a dose of 25 mg/kg (qd × 9) was found to give only a 34% ILS as compared with a 48% ILS for MTX at 1.3 mg/kg on the same schedule. Thus, α-amidation of MTX resulted in substantial loss of potency *in vivo* as well as *in vitro*. The decreased DHFR binding of (VII.11) in comparison with MTX was confirmed in experiments using *L. casei* enzyme [242]. The IC_{50} values of these compounds against *L. casei* DHFR were found to be 2.7 and 270 nM, respectively. Apparent K_i values were estimated from the equation $K_i = (K_m)(IC_{50})/[S]$ where [S] was the dihydrofolate concentration and K_m, the half-maximal velocity for dihydrofolate reduction, was taken to be 0.36 μM. Using this approximate method, (VII.11) was estimated to have a K_i of 1 nM as compared to 0.01 nM for MTX.

The effect of α-esterification and α-amidation on the binding of MTX to TS has also been evaluated [184]. While MTX was found to have a K_i of 30 μM against purified human enzyme, the estimated K_i values for the α-*n*-butyl ester (VII.6) and α-amide (VII.11) were 220 and 130 μM, respectively, demonstrating that blocking of the α-COOH group leads to a 5- to 10-fold decrease in binding.

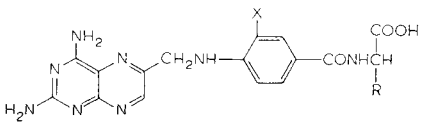
CHANGES IN REGION G

Five types of structural analogue embodying changes in Region G are the subject of this section: (a) compounds in which glutamic acid is replaced by α- or β-aminomonocarboxylic acids; (b) compounds in which glutamic acid is replaced by α- or β-aminodicarboxylic acids; (c) compounds in which glutamic acid is replaced by aminomonosulphonic or aminomonophosphonic acids; (d) compounds in which glutamic acid is substituted on the α, β- of γ-carbon; (e) compounds in which the γ-COOH group of the glutamate moiety is replaced by other acidic groups; (f) compounds in which the γ-COOH group of the glutamate moiety is blocked as an ester, amide or peptide; (g) compounds in which glutamic acid is replaced by α,ω-diamino monoacids and their N^{ω}-substituted derivatives; and (h) compounds with miscellaneous side-

chains. It should be noted that, while modification of Region G in classical antifolates has not, thus far, yielded any clinical candidates, a number of findings with these analogues have added to our general understanding of the mode of interaction of antifolates with dihydrofolate reductase and other enzymes of the folate pathway.

REPLACEMENT OF GLUTAMIC ACID BY α- OR β-AMINO MONOACIDS

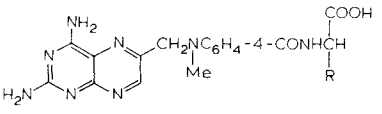
Studies in this area were reported as early as 1949 by Lederle chemists [243], who used the Waller synthesis (2,4,5,6-tetraaminopyrimidine, 2,3-dibromopropionaldehyde and an *N*-(4-aminobenzoyl)-α-amino acid or ester) to prepare the alanine, valine, isoleucine, serine, threonine, phenylalanine and tryptophan analogues (VIII.1)–(VIII.7), respectively. Compounds (VIII.2) and (VIII.3) were also treated with Cl_2 in AcOH to obtain the 3′-chloro derivatives (VIII.8) and (VIII.9). Although the data reported were very scanty, consisting only of activity ratios relative to 10-methylfolic acid in the *S. faecium* microbioassay, replacement of the glutamate moiety by α-amino monoacids was clearly shown to be an unpromising route to potent antifolates.



(VIII.1) R = CH_3, X = H
(VIII.2) R = $CH(CH_3)_2$, X = H
(VIII.3) R = $CH(CH_3)CH_2CH_3$, X = H
(VIII.4) R = CH_2OH, X = H
(VIII.5) R = $CH(CH_3)OH$, X = H
(VIII.6) R = $CH_2C_6H_5$, X = H
(VIII.7) R = C_9H_8N, X = H
(VIII.8) R = $CH(CH_3)_2$, X = Cl
(VIII.9) R = $CH(CH_3)CH_2CH_3$, X = Cl
(VIII.10) R = X = H

Other compounds with α-COH groups and neutral side-chains were described in 1978 [244] in the form of the MTX analogues (VIII.11)–(VIII.16). Of these, (VIII.12)–(VIII.14) were prepared in DL form, while (VIII.15) and (VIII.16) were said to be L-enantiomers. Appropriate *N*-[4-(*N*-methylamino)benzoyl]amino acids were prepared efficiently in several steps from 4-(*N*-Cbz-*N*-methylamino)benzoyl chloride and amino acid ethyl esters, and were condensed with 2,4-diamino-6-chloromethylpteridine, but unfortunately the yields in the final coupling step were $< 10\%$. The acute toxicity of (VIII.11)–(VIII.16) was found to be quite low (200–1000 mg/kg), but antitumour activity was likewise negligible except for a modest effect on Walker 256 rat carcinoma (as measured by tumour weight) with (VIII.13) and (VIII.16). It may be mentioned that similarly negative results against L1210

leukaemia had already been reported independently [245] for a series of amino-acid methyl and ethyl esters including those of DL-2-aminobutyric acid, L-leucine, DL-norleucine, L-methionine, L-tryptophan, L-tyrosine and L-proline. It was assumed in this study that nonspecific serum esterases in the mouse would rapidly cleave the esters to the acids, as had been observed with esters of MTX [237].



(VIII.11) R = H
(VIII.12) R = CH_3
(VIII.13) R = CH_2CH_3
(VIII.14) R = $CH(CH_3)_2$
(VIII.15) R = $CH_2CH(CH_3)_2$
(VIII.16) R = $CH_2C_6H_5$

The biological properties of the N^{10}-unsubstituted glycine derivative (VIII.10) were examined in some detail [14] as part of a larger effort directed toward side-chain-modified MTX and AMT analogues. Compound (VIII.10) was prepared in 55% yield by condensation of 2,4-diamino-6-bromomethylpteridine with *N*-(4-aminobenzoyl)-glycine.

The IC_{50} of (VIII.10) as an inhibitor of DHFR from pigeon liver was found to be 1.1 μM as compared with 0.026 μM for AMT [14]. Removal of the entire CH_2CH_2COOH side-chain thus led to a substantial loss of binding. Most of this loss is probably due to removal of the CH_2CH_2 chain rather than loss of the COOH group, since γ-esterification and γ-amidation have a relatively minor effect on binding (*vide infra*). In assays against KB human epithelial carcinoma cells in culture, (VIII.10) had an IC_{50} value of 220 μM, while AMT had an IC_{50} of 0.75 μM. Thus, while (VIII.10) was 40-fold less tightly bound to avian DHFR than AMT, its ability to inhibit the growth of mammalian cells was diminished 20,000-fold, suggesting that side-chain deletion caused also a profound change in the ability of the drug to enter cells. Consistent with this was the finding that, in assays against L1210 leukaemia in mice, (VIII.10) was inactive at 80 mg/kg (qd × 9) whereas AMT on the same schedule gave a 74% ILS at 0.67 mg/kg.

Folic and homofolic acid analogues with the glutamic acid moiety replaced by glycine and DL-alanine were described [72] as part of a larger series consisting mainly of compounds with dicarboxylic acid side-chains (*vide infra*). N^2-Acetyl-N^{10}- trifluoroacetylpteroic acid and N^2 -acetyl-N^{10}-trifluoroacetylhomopteroic acid was each activated with isobutyl chloroformate, the mixed anhydride was condensed with ethyl glycinate or ethyl DL-alaninate, and all

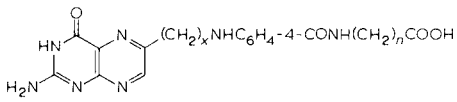
blocking groups were removed at the same time by treatment with 0.2 M NaOH at 100 °C for 45 min to obtain low yields of compounds (VIII.17)–(VIII.20), respectively. Chemical reduction with sodium dithionite in the presence of 2-mercaptoethanol yielded the corresponding 7,8-dihydro derivatives, which on enzymatic reduction with DHFR were converted to 5,6,7,8-tetrahydro derivatives with the 'natural' configuration at position 6. Interestingly, the dihydrofolate analogues H_2-(VIII.17) and H_2-(VIII.18) were reduced at a slightly higher rate by DHFR than was dihydrofolate itself; however, while the dihydrohomofolate analogue H_2-(VIII.19) was reduced at about half the rate of dihydrofolate, the rate of reduction of H_2-(VIII.20) was higher than that of dihydrofolate by about 50%, and was higher than that of H_2-(VIII.17) or H_2-(VIII.18). The tetrahydrofolate analogues H_4-(VIII.17) and H_4-(VIII.18) were not found to have any substrate or inhibitor activity for TS, while the tetrahydrohomofolate analogues H_4-(VIII.19) and H_4-(VIII.20) were found to have only slight inhibitory activity in comparison with compounds in which two carboxyl groups were present in the side-chain (*vide infra*). These results established for the first time that a terminal carboxyl group on the folate/antifolate side-chain is critical for binding to TS.

(VIII.17) n = 1, R = H (VIII.20) n = 2, R = Me
(VIII.18) n = 1, R = Me (VIII.21) n = 1, R = Et
(VIII.19) n = 2, R = H

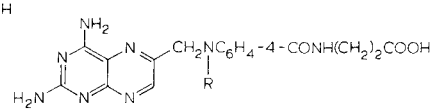
In a recent study [246], the 2-aminobutyric acid analogue (VIII.21) was synthesized and reduced to H_4-(VIII.21), which was tested as an inhibitor of hog liver folylpolyglutamate synthetase. The K_i for inhibition of (6*S*)-tetrahydrofolate polyglutamylation was found to be 68 μM, a value approximately 10-fold higher than the substrate K_m for (6*S*)-tetrahydrofolate. This K_i/K_m ratio may be viewed as reflecting the deletion of the γ-carboxyl group from the side-chain.

β-Alanine analogues of AMT and MTX [243, 244], as well as those of reduced folic and homofolic acids [72], have been prepared via standard chemical routes in order to assess the importance of the α-carboxyl group for binding to enzymes of the folate pathway. Plante *et al.* [72] found the folic and homofolic acid analogues H_2-(VIII.22) and H_2-(VIII.23) to have only about 30–40% of the substrate activity of the corresponding glutamate analogues toward DHFR, with the activity of H_2-(VIII.23) being similar to that of the

glycine derivative H_2-(VIII.19). The enzymatically formed H_4-(VIII.23) was a weak inhibitor of TS, with only about 15% of the activity of tetrahydrohomofolate at saturating concentrations. Wright *et al.* [243] briefly cited the AMT analogue (VIII.26) but gave no biological data. More recently, other workers [244] synthesized the MTX analogue (VIII.27) and reported it to be inactive against L1210 leukaemia in mice at a dose of 25 mg/kg (qd × 6).



(VIII.22) $x = 1, n = 2$
(VIII.23) $x = 2, n = 2$
(VIII.24) $x = 1, n = 3$
(VIII.25) $x = 1, n = 4$

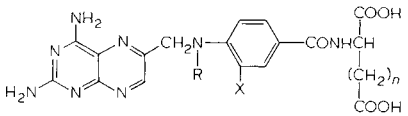


(VIII.26) R = H
(VIII.27) R = Me

In a recent study assessing the structural specificity of folate analogues as substrates/inhibitors of purified folylpolyglutamate synthetase (FPGS) from hog liver, *N*-pteroyl-4-aminobutanoic acid (VIII.24), *N*-pteroyl-5-aminopentanoic acid (VIII.25) and their reduced derivatives H_2-(VIII.24), H_4-(VIII.24), H_2-(VIII.25) and H_4-(VIII.25) were synthesized [246]. Standard procedures similar to those described by other workers [72, 247] were followed, but yields and other chemical data were not given. The tetrahydro compounds were also converted to 5,10-methylene derivatives and 10-formyl derivatives. When assayed at a concentration of 100 μM, (VIII.24) had 21% of the substrate activity of 50 μM (6*RS*)-tetrahydrofolate, while 100 μM (VIII.25) was much less active. The substrate activity of 50 μM (VIII.24) was only 4% of that of 50 μM (6*R*,6*S*)-tetrahydrofolate. When 50 μM H_4-(VIII.24) and 50 μM H_4-(VIII.25) were compared, the aminobutanoic acid again proved to be the better substrate. Kinetic constants (K_m, μM) for the above compounds as FPGS substrates were found to be: (VIII.24), 810; (VIII.25), 1090; H_2-(VIII.24), 12; H_2-(VIII.25), 22; H_4-(VIII.24), 74; H_4-(VIII.25), 163. The K_m for (6*S*)-tetrahydrofolate was 7.7 μM. Thus, the dihydro analogues were better substrates than either the aromatic or tetrahydro analogues. Moreover, rather remarkably in view of the absence of an α-carboxyl group, these compounds had a K_m only 2- to 3-fold different from that of the natural substrate (6*S*)-tetrahydrofolate. 4-Aminobutanoic and 5-aminopentanoic acids can be regarded in the context of this study as α-decarboxylated analogues of glutamic and 2-aminoadipic acid, respectively.

REPLACEMENT OF GLUTAMIC ACID BY α- OR β-AMINO DIACIDS

The earliest paper describing MTX or AMT analogues containing amino diacids other than glutamic acid appeared in 1949 from the Lederle group [243]. Among the compounds obtained in this work via the Waller synthesis was the malonic acid derivative (VIII.28). In microbioassays against *S. faecium*, (VIII.28) was found to be 300-fold less inhibitory than 10-methylfolic acid. The L-aspartate analogue (VIII.29) (once known as 'Amino-ANFOL') was also prepared via the Waller process and converted to the 3'-chloro derivative (VIII.30), which had about 5% of the inhibitory activity of 10-methylfolic acid [243]. It was evident from these results that reduction of the distance between the carboxyl groups in the side-chain was very detrimental to biological activity, at least in the pteridine series. This low level of activity in comparison with MTX led to discontinuation of further work on chain-shortened analogues of AMT. An interesting recent observation [94], however, was that (VIII.29) possesses some activity as an inhibitor of folylpolyglutamate synthetase from mouse liver. With 500 μM folic acid and 1 mM L-glutamic acid as co-substrates, 500 μM (VIII.29) inhibited the glutamylation reaction by 60%. Substrate activity, on the other hand, was negligible (< 0.5% of the activity of folic acid).



(VIII.28) X = H, R = Me, n = 0
(VIII.29) X = R = H, n = 1
(VIII.30) X = Cl, R = H, n = 1
(VIII.31) X = H, R = Me, n = 1

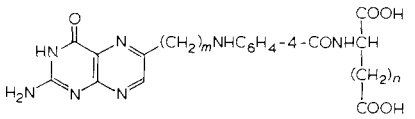
The synthesis as well as biochemical and pharmacological properties of the historically important L-aspartate analogue of MTX, (VIII.31), were described in detail [248] in 1965. In experiments using DHFR from L1210/FR8 cells, an MTX-resistant line originally derived by treatment of leukaemic mice with DCM [81], (VIII.31) was shown to be a competitive inhibitor, with an estimated K_i of 2 nM. The method used to estimate the K_i was the same as the one that Werkheiser [249] and subsequently Bertino *et al.* [250] had used to obtain estimated K_i values of 0.03 and 0.67 nM, respectively, for MTX. The enzyme titration curve for (VIII.31) showed greater curvilinearity than that of MTX, and appeared to level off at about 80–90% inhibition.

This suggested that dissociation of the enzyme-inhibitor complex was easier when the length of side-chain was decreased by one carbon. In contrast to MTX, therefore, (VIII.31) could not be classified as a 'stoichiometric' inhibitor. The decreased potency of (VIII.31) relative to MTX as a DHFR inhibitor was quite accurately reflected in a decreased ability to inhibit purine synthesis as measured by [^{14}C]formate incorporation into the acid-insoluble fraction of L1210 cells grown i.p. in mice. Whereas treatment with 0.1 mg/kg of MTX led to a 50% reduction in labeling, a similar effect with (VIII.31) could be achieved only at a 40- to 50-fold higher dose. In experiments measuring DHFR activity in mouse liver after treatment with single 10 mg/kg doses of drug, only 17% residual activity remained in comparison with untreated controls when the animals were treated with MTX, whereas activity remained unaffected in animals treated with (VIII.31). Even when four 10 mg/kg doses of (VIII.31) were administered, residual activity remained high (85%). It was evident from these results that (VIII.31) was much less toxic than MTX to liver cells, which could perhaps be therapeutically advantageous. However, when *in vivo* tests were conducted against L1210 leukaemia in mice, (VIII.31) proved to be far less effective than MTX. Thus, daily treatment with 50 mg/kg of (VIII.31) produced only a 45% increase in mean lifespan, whereas daily treatment with 0.78 mg/kg of MTX caused half the animals to survive at least five times as long as untreated controls. In experiments using the MTX-resistant L1210/FR8 tumour, (VIII.31) was no more effective than against the wild-type tumour. The historical significance of this compound was acquired not so much from its biological activity, which was disappointing, but from the fact that it could be viewed as the structural ancestor of a much more interesting series of quinazoline antifolates containing L-aspartate rather than L-glutamate as the side-chain (*vide infra*).

Because it seemed possible that insertion of extra CH_2 groups in the amino-acid side-chain of an antifolate might be less detrimental than their deletion, DL-2-aminoadipic and DL-2-aminopimelic acids were used by Plante *et al.* [72] to prepare folic and homofolic acid analogues (VIII.32)–(VIII.35) by standard mixed anhydride condensation reactions as described in the preceding section. The corresponding 7,8-dihydro derivatives H_2-(VIII.32)–H_2-(VIII.35) were obtained by chemical reduction with sodium dithionite, while the 5,6,7,8-tetrahydro derivatives H_4-(VIII.32)–H_4-(VIII.35) were prepared from the dihydro compounds by enzymatic reduction with DHFR. The aminoadipate analogues H_2-(VIII.32) and H_2-(VIII.34) were approximately equivalent to dihydrofolate as DHFR substrates, but interestingly, the aminopimelate analogues H_2-(VIII.33) and H_2-(VIII.35) both proved to be approximately 2-fold better substrates, suggesting that elongation of the side-chain

might favour binding to the enzyme. In assays of TS inhibitor activity, H_4-(VIII.33) and H_4-(VIII.35) proved to be about 60% as effective as tetrahydrohomofolate, and were the best among some twenty amino-acid variants tested.

In recent work, the interaction of (VIII.32) and (VIII.33) with hog liver FPGS, these compounds were found to be neither substrates nor inhibitors, although H_4-(VIII.33) did have some substrate activity with FPGS from *Corynebacterium* [246]. The latter observation was noteworthy in view of the report by Moran *et al.* [94] that the 2-aminopimelate analogue of MTX (*vide infra*) similarly showed modest substrate activity for FPGS from mouse liver.



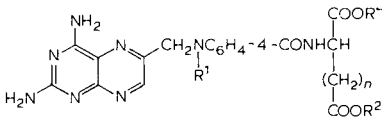
(VIII.32) $m = 1, n = 3$ (VIII.34) $m = 2, n = 3$
(VIII.33) $m = 1, n = 4$ (VIII.35) $m = 2, n = 4$

Chain-lengthened DL-2-aminoadipic and DL-2-aminopimelic acid analogues of MTX and AMT have been reported by several investigators [14, 251–253]. The MTX analogues (VIII.36) and (VIII.37), in the DL-form, were obtained first in poor yield via a Waller-type synthesis [251], and subsequently from 4-amino-4-deoxy-N^{10}-methylpteroic acid by mixed anhydride synthesis of the diethyl esters (VIII.38) and (VIII.39) followed by alkaline hydrolysis [252, 253]. The AMT analogue (VIII.40), likewise in the DL-form, was prepared from 2,4-diamino-6-bromomethylpteridine and DL-2-aminopimelic acid [14]. In assays against DHFR from pigeon liver, (VIII.40) was found to have an IC_{50} of 0.013 μM as compared with 0.026 μM for AMT, suggesting that chain elongation was well tolerated by the enzyme and, in fact, seemed to favour binding [14]. However, in assays against cultured KB cells the IC_{50} value of (VIII.40) was 8-fold higher than that of MTX and *in vivo* against L1210 leukaemia in mice only a 57% ILS was obtained at a dose of 20 mg/kg (qd × 9), whereas MTX at 0.67 mg/kg on the same schedule gave a 74% ILS.

Because of the possibility that diesters of chain-extended analogues might be more effective than the diacids, Rosowsky *et al.* [253] examined the *in vitro* and *in vivo* biological activity of the diethyl esters [VIII.38) and (VIII.39) relative to that of the diethyl ester of L-MTX. In assays against *S. faecium* and human leukaemic lymphoblasts (CEM cells) in culture, these chain-extended diesters proved to be approximately half as active as the L-MTX diester. It

thus appeared (if one allowed for the possibility that only the L-enantiomers were active) that (VIII.38), (VIII.39) and the diester of MTX were approximately equipotent as inhibitors of bacterial as well as mammalian cell growth. When tested against L1210 leukaemia in mice, (VIII.38) gave a 44% ILS at a dose of 120 mg/kg (q3d × 3) and (VIII.39) gave a 67% ILS on the same dose schedule. The diethyl ester of MTX at its maximally tolerated dose of 45 mg/kg likewise gave a 44% ILS. The three esters were therefore similar in therapeutic efficacy, although higher doses of the chain-extended DL-compounds had to be given.

In order to determine whether the presence of D-enantiomers in the chain-extended MTX analogues might be masking the true activity of the L-enantiomers, Rosowsky *et al.* [253] synthesized the L-enantiomers of (VIII.36) and (VIII.37), and also prepared the L-enantiomer of the previously unknown 2-aminosuberic acid analogue, (VIII.41). Condensation of 4-amino-4-deoxy-N^{10}-methylpteroic acid (MeAPA) with diethyl L-2-aminoadipate, di-*t*-butyl L-2-aminopimelate, and dimethyl L-2-aminosuberate by the diethyl phosphorocyanidate method afforded the diesters (VIII.42) (42%), (VIII.43) (71%), and (VIII.44) (85%), respectively. Ester groups were then removed with $Ba(OH)_2$ ((VIII.42), (VIII.44)) or trifluoroacetic acid ((VIII.43)) to form the desired L-diacids. In a spectrophotometric assay of DHFR inhibition using enzyme from L1210/R71 cells, the L-diacids were all found to have IC_{50} values in the 35–40 nM range as compared with 32 nM for MTX, confirming that chain elongation was well tolerated for binding to the enzyme and was minimally affected by the number of CH_2 groups. In growth inhibition assays using L1210 cells, on the other hand, activity appeared to increase with chain



(VIII.36) R^1 = Me, R^2 = H, n = 3
(VIII.37) R^1 = Me, R^2 = H, n = 4
(VIII.38) R^1 = Me, R^2 = Et, n = 3
(VIII.39) R^1 = Me, R^2 = Et, n = 4
(VIII.40) R^1 = R^2 = H, n = 4
(VIII.41) R^1 = Me, R^2 H, n = 5
(VIII.42) R^1 = Me, R^2 = Et, n = 3
(VIII.43) R^1 = Me, R^2 = Et, n = 4
(VIII.44) R^1 = Me, R^2 = Et, n = 5
(VIII.45) R^1 = Me, R^2 = Et, n = 6
(VIII.46) R^1 = Me, R^2 = Et, n = 7
(VIII.47) R^1 = Me, R^2 = Et, n = 8
(VIII.48) R^1 = Me, R^2 = Et, n = 9
(VIII.49) R^1 = Me, R^2 = Et, n = 10
(VIII.50) R^1 = Me, R^2 = H, n = 6
(VIII.51) R^1 = Me, R^2 = H, n = 7
(VIII.52) R^1 = Me, R^2 = H, n = 8
(VIII.53) R^1 = Me, R^2 = H, n = 9
(VIII.54) R^1 = Me, R^2 = H, n = 10
(VIII.55) R^1 = CHO, R^2 = Et, n = 6
(VIII.56) R^1 = CHO, R^2 = Et, n = 9
(VIII.57) R^1 = CHO, R^2 = Et, n = 10
(VIII.58) R^1 = R^2 = H, n = 6
(VIII.59) R^1 = R^2 = H, n = 9
(VIII.60) R^1 = R^2 = H n = 10

length. The IC_{50} value of L-(VIII.36) was found to be 0.03 μM (versus 0.02 μM for MTX) while that of L-(VIII.37) was 0.01 μM and that of L-(VIII.41) was 0.006 μM. Thus, insertion of three extra CH_2 groups in the side-chain brought about a 3-fold increase in potency in cell culture, suggesting the possibility of improved uptake by the cells. *In vivo* against L1210 leukaemia in mice, compound (VIII.36) given at a dose of 60 mg/kg (qd × 9) produced a 133% ILS, while the longer homologues (VIII.37) and (VIII.41) gave about the same prolongation of survival at a lower dose of 40 mg/kg. On a more frequent dosing schedule (bid × 10), (VIII.36) gave a 133% ILS at 15 mg/kg. The potency of MTX was also determined for comparison, and, as expected, was found to be schedule-dependent. On a q2d × 5 schedule, 15 mg/kg of MTX gave a 122% ILS, whereas on a qd × 9 schedule a 133% ILS was obtained at 6 mg/kg and on a bid × 10 schedule a 112% ILS was obtained at 0.75 mg/kg. Overall it appeared that these chain-extended analogues were capable of achieving the same therapeutic effect as MTX, but the total dose administered, regardless of the schedule, was always higher.

The availability of compounds (VIII.36), (VIII.37) and (VIII.41) provided an opportunity to systematically examine the effect of chain length on the ability of these molecules to influence folate/antifolate transport, as well as on their ability to interact with folate-specific enzymes involved in cell growth and replication. In a study of the interaction of several MTX analogues with human TS purified from cells of leukaemic patients [182], the ability of DL-(VIII.36) and DL-(VIII.37) to competitively inhibit the formation of dTMP from dUMP and 5,10-methylenetetrahydrofolate was compared. While the estimated K_i for MTX in this assay was 30 μM, that of the chain-extended compounds was 62 to 40 μM, respectively. Therefore, if one allowed for the possibility that only the L-enantiomers bound efficiently to the active site of TS (L-(VIII.36) was not available at the time of this work), the insertion of one or two CH_2 groups in the side-chain appeared to favour binding, if only to a small degree. In another investigation [254], the ability of leucovorin to protect two human lymphoid cell lines (CEM and LAZ-007) from the toxic effects of L-(VIII.39), MTX, and the lipid-soluble nonclassical antifolates DDMP and trimetrexate (TMQ) was compared. While leucovorin was 40–50-times more effective in protecting LAZ-007 cells than CEM cells from the toxic effects of MTX, there was very little 'differential protection' when DDMP or TMQ was used as the antifolate. The aminoadipate analogue L-(VIII.36) was similar to MTX, in that leucovorin protection was more readily achieved in the LAZ-007 cells. This suggested that cellular uptake of L-(VIII.36) might be primarily by active transport. To examine this point further, the authors compared the ability of L-(VIII.36) and leucovorin to

inhibit [^{3}H]MTX influx into CEM cells. Competitive inhibition kinetics were observed for both compounds, but the K_i for leucovorin was found to be 2.1 μM (K_i/K_m = 1.5), while the K_i for L-(VIII.36) was 15 μM (K_i/K_m = 11). Thus, it appears that L-(VIII.36) is probably recognized as a substrate by the membrane carrier for reduced folates, but is less tightly bound than either MTX or leucovorin. As expected the IC_{50} value for inhibition of both CEM and LAZ-007 cells by L-(VIII.36) was somewhat higher than the IC_{50} value for MTX.

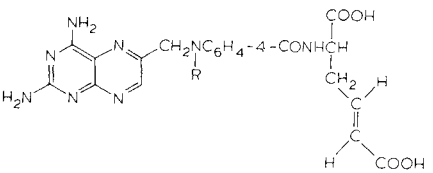
All the *in vitro* and *in vivo* data accumulated with the chain-lengthened analogues of MTX pointed to the likelihood that an important determinant of their lower molar potency was their inability to form polyglutamates. This was confirmed in a study [94] in which a number of MTX analogues were tested as substrates and inhibitors of partly purified FPGS from mouse liver. The substrate activity of the aminoadipate and aminosuberate analogues L-(VIII.36) and L-(VIII.38) was found to be only 1–2% of that of folic acid under identical assay conditions. On the other hand, the aminopimelate analogue L-(VIII.37) did show some activity (20% of that of folic acid). It appeared that when two CH_2 groups were added, the side-chain could adopt a conformation that allowed the terminal carboxyl group to fit properly into the FPGS active site, whereas when either one or three CH_2 were added this was not possible. Although L-(VIII.36) and L-(VIII.38) had negligible substrate activity, they were weak competitive inhibitors, as was L-(VIII.37). Polyglutamylation of folic acid was inhibited in the presence of an equal concentration of any of the chain-lengthened analogues by 25–35%. As mentioned above, the L-aspartate analogue of AMT, (VIII.30), was a very poor FPGS substrate, but was a moderately active inhibitor.

Most recently Rosowsky *et al.* [255] reported the synthesis and biological activity of a series of DL-α-amino-α,γ-alkanedioic acids containing six to ten CH_2 groups. The impetus for this work was a desire to learn whether lengthening of the side-chain beyond five CH_2 groups would lead to a continued increase in potency as had been observed with the aminoadipate, aminopimelate and aminosuberate analogues. 4-Amino-4-deoxy-N^{10}-methylpteroic acid was condensed with appropriate diethyl esters in the presence of diethyl phosphorocyanidate, and the resulting products ((VIII.45)–(VIII.49)) were hydrolyzed with $Ba(OH)_2$ to form the diacids (VIII.50)–(VIII.54). Similarly obtained from 4-amino-4-deoxy-N^{10}-formylpteroic acid were the diesters (VIII.55)–(VIII.57) and diacids (VIII.58)–(VIII.60). All the DL-diacids were found to be potent inhibitors of DHFR from L1210/R81 cells, with IC_{50} values of 0.023–0.034 μM for the MTX analogues (VIII.50)–(VIII.54) and 0.054–0.067 μM for the AMT analogues (VIII.58)–(VIII.60). The chain-leng-

thened compounds were also inhibitors of folylpolyglutamate synthetase from mouse liver (40–60% inhibition at equimolar concentrations of folic acid as the substrate). The IC_{50} values for inhibition of cell growth in culture by the MTX analogues were 0.016–0.64 μM against CEM human leukaemic lymphoblasts and 0.0012–0.026 μM against L1210 murine leukaemia cells. The MTX analogues with six CH_2 groups (DL-(VIII.50)) was, as hoped, more potent than the aminosuberate analogue L-(VIII.41), but activity thereafter began to decrease. The optimal chain length for growth inhibition also seemed to be species-dependent, with CEM cells being inhibited best by the compound with nine CH_2 groups (DL-(VIII.53)), while L1210 cells were most sensitive to the one with six CH_2 groups (DL-(VIII.50)). Among the AMT analogues, on the other hand, the most active compound against L1210 cells was the one with nine CH_2 groups (DL-(VIII.53)), which had an IC_{50} value of 0.00065 μM as compared with 0.0046 μM for MTX and 0.002 μM for AMT. If one allows for the fact that DL-(VIII.53) is a mixture of D- and L-enantiomers, it is very possible that the L-enantiomer would be as much as 10-fold more potent than MTX, at least in cell culture. In assays of antitumour activity against L1210 leukaemia in mice on a qd × 9 schedule, on the other hand, the most active of the MTX analogues turned out to be the one with ten CH_2 groups (DL-(VIII.54)), which gave a 137% ILS at 20 mg/kg. The analogue with six CH_2 groups (DL-(VIII.50)), which was more potent than DL-(VIII.54) *in vitro*, was less potent *in vivo*, giving a 105% ILS at 60 mg/kg. The AMT analogues, despite their high activity *in vitro*, were less effective than the MTX analogues *in vivo* because of increased host toxicity. The fact that DL-(VIII.54) and some of the other chain-lengthened analogues gave approximately the same prolongation of lifespan *in vivo* as MTX is of interest because these compounds do not form polyglutamates and are therefore unlikely to be retained very long in tissues such as liver, where MTX polyglutamates may linger for weeks or months after administration of the parent drug. It has been suggested [253, 255] that non-polyglutamylated MTX analogues deserve consideration in the long-term low dose treatment of chronic disorders like psoriasis [256] and rheumatoid arthritis [257]. It has also been proposed [253, 255] that non-polyglutamated MTX analogues may be useful in the treatment of MTX-resistant tumours whose capacity to polyglutamylate MTX is impaired in comparison with that of normal tissues of the host. Several tumour cell lines with an apparent defect in FPGS activity have been described [258–263).

MTX and AMT analogues with a double bond in the glutamic acid side-chain are unknown, but their chain-extended analogues DL-(VIII.61) and DL-(VIII.62) have been described recently [219]. The synthesis of these

compounds was achieved from diethyl DL-2-amino-3-*trans*-hexenedioate by condensation with 4-amino-4-deoxy-N^{10}-methylpteroic and 4-amino-4-deoxy-N^{10}-formylpteroic acid, respectively, followed by alkaline hydrolysis. The starting amino acid was obtained by addition of ethyl bromocrotonate to the anion generated on treatment of the benzophenone imide of ethyl glycinate with lithium diethylamide. An interesting feature of DL-(VIII.61) and DL-(VIII.62) is that the *trans* geometry of the double bone causes the side-chain to be shorter and conformationally more restricted than would be the case in a saturated derivative. Compounds DL-(VIII.61) and DL-(VIII.62) were found to be potent DHFR inhibitors, with IC_{50} values of 0.032 and 0.046 μM, respectively, as compared with a previously determined IC_{50} value of 0.039 μM for the saturated analogue L-(VIII.36). It has been shown that L-MTX is 10-times more inhibitory than D-MTX against DHFR [264]. On this basis, it is conceivable (but by no means assured) that the IC_{50} values of L-(VIII.61) and L-(VIII.62) lie in the 15–25 μM range. However, while it would be interesting if the introduction of a *trans* double bond had a favourable effect on DHFR binding, this cannot be definitively established without carrying out an unequivocal synthesis of the enantiomerically pure ligands.

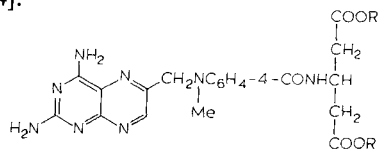


(VIII 61) R = Me
(VIII 62) R = H

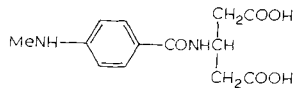
Replacement of L-glutamic acid in MTX by DL-3-aminoglutaric acid to give the analogue DL-(VIII.63) has been shown to substantially decrease biological activity, demonstrating once again (*vide supra*) the critical importance of an α-carboxyl group in antifolates. The first synthesis of DL-(VIII.63), via the diethyl ester DL-(VIII.64), involved a Waller-type synthesis from *N*-[4-(*N*-methylaminobenzoyl)amino]-DL-3-aminoglutaric acid (DL-(VIII.65)), 2,4,5,6,-tetraaminopyrimidine, and 2,3-dibromopropionaldehyde [196]. Attempted synthesis of DL-(VIII.64) from the amino-acid diester and 4-amino-4-deoxy-N^{10}-methylpteroic acid in the presence of DCC was thwarted by extensive acylurea formation, but would probably have succeeded if some other coupling reagent (*e.g.*, diethyl phosphorocyanidate) had been used. Compound DL-(VIII.65) was synthesized from 4-(*N*-formyl-*N*-methylamino)benzoyl chloride by condensation with diethyl DL-3-aminoglutarate, followed by alkaline hydrolysis of the ester groups and acid hydrolysis of the

N-formyl group. Subsequently, DL-(VIII.64) was prepared by a modified Taylor synthesis by Rosowsky *et al.* [265]. 4-(*N*-Methyl-*N*-tosylamino)benzoyl chloride was condensed with diethyl DL-3-aminoglutarate, the tosyl group was removed with HBr, the resultant amine was coupled to 2-amino-5-chloromethyl-3-cyanopyrazine *N*-oxide [24], and the *N*-oxide was directly reduced to amino nitrile DL-(VIII.66) with triethyl phosphite and condensed with guanidine carbonate to form diester DL-(VIII.64) in low yield.

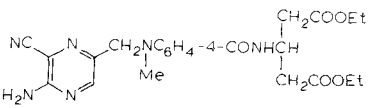
Lee *et al.* [196] reported DL-(VIII.63) to have an IC_{50} value of 10 nM against DHFR from L1210 mouse leukaemia cells, while MTX had an IC_{50} value of 3.5 nM against this enzyme. This result was somewhat surprising, since it suggested that moving the amino group from the α- to the β-carbon caused only a 3-fold decrease in DHFR. *In vivo* assays against L1210 leukaemia in mice gave results somewhat inconsistent with the enzyme inhibition data, in that treatment with 100 mg/kg (qd × 9) led only to a statistically non-significant 18% ILS, while MTX at 2 mg/kg on the same schedule gave a 54% ILS. An increase in the dose of DL-(VIII.63) to 400 mg/kg killed all the mice, suggesting that intermediate doses, or perhaps a more frequent dosing schedule, might have given better results. In the light of what is known today about the important role of polyglutamylation as a determinant of *in vivo* activity, it is not surprising that DL-(VIII.63) was much less active than MTX. The activity of DL-(VIII.63) as a substrate for FPGS from mouse liver has, in fact, been shown to be $< 0.01\%$ of that of folic acid [94].



(VIII.63) R = H
(VIII.64) R = Et



(VIII.65)

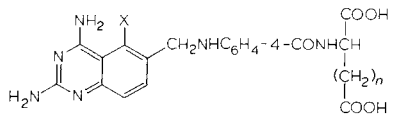


(VIII.66)

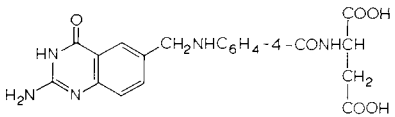
While the L-glutamic acid moiety in MTX and AMT has been replaced by several amino dicarboxylic acids as discussed above, this type of structure modification has been practised only sparingly with MTX or AMT analogues concomitantly modified in Regions A–E. Indeed, the only substantial body of

work done along these lines, thus far, has been in the quinazoline series. Although this work is not often cited any more, it is still worth recalling, not only because of its historical importance, but also because many of the basic concepts formulated during the course of biochemical and pharmacological studies on these quinazoline analogues two decades ago continue to be relevant today. Furthermore, it is not impossible, in the context of knowledge gained since these original studies, that aspartates other than those of the quinazoline family would prove worthwhile to examine for antifolate activity.

Among the compounds synthesized by Davoll and Johnson [40] as part of their pioneering work on 5,8-dideaza analogues were the malonic acid derivatives (VIII.67)–(VIII.69) and the L-aspartic acid derivatives (VIII.70)–(VIII.73). Of these, (VIII.67)–(VIII.72) are chain-shortened analogues of 5,8-dideazaAMT, while (VIII.73) is an analogue of 5,8-dideazafolic acid. Diethyl *N*-(4-aminobenzoyl)malonate [243] and diethyl *N*-(4-aminobenzoyl)-L-aspartate were reductively coupled to the appropriate quinazoline-6-carboxaldehyde or quinazoline-6-carbonitrile, and ester groups were cleaved by treatment with NaOH in aqueous ethanol at room temperature. For the synthesis of diethyl *N*-(4-aminobenzoyl)-L-aspartate, the amino acid diester was *N*-acylated with 4-nitrobenzoyl chloride and the amino group was reduced (H_2-Pd-C). The same reaction sequence was followed to obtain diethyl *N*-(4-aminobenzoyl)-D-aspartate, which was used to prepare quinazoline analogues with the side-chain in the D-configuration.



(VIII.67) X = H, *n* = 0 (VIII.70) X = H, *n* = 1
(VIII.68) X = Me, *n* = 0 (VIII.71) X = Me, *n* = 1
(VIII.69) X = Cl, *n* = 0 (VIII.72) X = Cl, *n* = 1



(VIII.73)

The L-aspartate analogues (VIII.70)–(VIII.72), which also came to be known as quinaspar, methasquin, and chloroasquin [48], were found to be potent inhibitors of the growth of two L1210 cell lines in culture by Hutchison [51]. Curiously enough, while they were less potent on a molar basis than the corresponding L-glutamates of the quinazoline series (*vide supra*), the L-aspartate analogues were nonetheless more potent than MTX. The IC_{50} value of the 5-unsubstituted compound (VIII.70) against L1210/TC_1 cells, for example, was 52 nM, while that of MTX was 237 nM. The 5-Me and 5-Cl derivatives (VIII.71) and (VIII.72) had IC_{50} values of 68 and 79 nM, and were

therefore somewhat less potent, as was also true in the L-glutamate series, where IC_{50} values for the 5-unsubstituted, 5-Me and 5-Cl compounds were 8.6, 12 and 11 nM, respectively. A rather surprising observation was also the fact that the D-enantiomer of (VIII.70), with an IC_{50} value of 49 nM, was essentially equipotent with the L-enantiomer. An explanation for this odd finding was not given. One might speculate that some racemization had perhaps occurred during the synthesis (for example, during ester hydrolysis in base), but even if the test sample were a 1:1 racemic mixture, it would be difficult to explain the results unless the two enantiomers had comparable activity. Data on the malonate analogues were not reported, and it must be assumed that they were inactive or very weakly active. The 2-amino-4(3*H*)-oxo derivative (VIII.73) has likewise been found to be of limited interest [52].

Potent *in vitro* antitumour activity with methasquin (VIII.71) has also been observed against C1300 murine neuroblastoma cells [266]. The IC_{50} value was found to be 0.008 μM as compared with 0.05 μM for MTX and 0.01 μM for 5,8-dideazaMTX. Again, this was a striking level of activity considering that the side-chain was L-aspartic acid rather than L-glutamic acid. In spectrophotometric assays of DHFR activity in crude extracts of the cells, methasquin and MTX gave IC_{50} values of 4 and 2 nM, respectively. This suggested that neuroblastoma cells might be able to accumulate methasquin more efficiently than MTX.

A comparison of the potencies of (VIII.70)–(VIII.72) as inhibitors of DHFR from rat liver and *S. faecium* was reported in 1977 by Hynes *et al.* [54]. The IC_{50} values of the three quinazolines as inhibitors of rat liver enzyme were found to be 1.7 nM for (VIII.70), 0.4 nM for (VIII.71), and 6.4 nM for (VIII.72). For the *S. faecium* enzyme, these IC_{50} values were 6.2, 2.5 and 2.8 nM respectively. Thus, methasquin was the best inhibitor of both enzymes. Interestingly, when the side-chain was L-glutamic acid, the best inhibitor of rat liver DHFR was 5-unsubstituted derivative (5,8-dideazaAMT) while the best inhibitor of *S. faecium* enzyme was 5-chloro-5,8-dideazaAMT. Structure–activity correlations among the L-asparate and L-glutamate derivatives were obviously quite subtle and difficult to interpret. Overall, however, the results were in qualitative agreement with those obtained earlier by Hutchinson *et al.* [267], who showed with enzyme from L1210 cells that all three quinazoline aspartates were potent antifolates and deserved to be tested for antitumour activity.

In vivo experiments using mice with L1210 mouse leukaemia were undertaken by Hutchison [51] at the Sloan-Kettering Institute in the late 1960's. Treatment with (VIII.70) (6 mg/kg, q2d × 8) afforded a 150% increase in lifespan (ILS), while the corresponding L-glutamate analogue on the same

treatment schedule gave only a 63% ILS at its highest tolerated dose of 0.2 mg/kg. Treatment with MTX (3 mg/kg, q2d × 8) likewise gave a 150% ILS. Thus, in the L1210 model, (VIII.70) and MTX were comparable in potency as well as therapeutic benefit. The D-enantiomer of (VIII.70), despite the fact that it was equipotent with the L-enantiomer *in vitro*, was less active *in vivo*, giving only a 63% ILS at 6 mg/kg. The 5-methyl derivate (VIII.71), with a 186% ILS at 3 mg/kg, was somewhat superior superior to either (VIII.70) or MTX, whereas the 5-chloro derivative (VIII.72), with a 150% ILS at 6 mg/kg, was only as good as (VIII.70). The fact that (VIII.71) was superior to (VIII.70) *in vivo*, even though it was less potent *in vitro*, suggested that the introduction of a 5-methyl substituent was perhaps bringing about a pharmacodynamically or pharmacokinetically favourable effect deserving of further investigation (*vide infra*).

Though it was no better against L120 leukaemia than its 5-unsubstituted congener, the 5-chloro derivative (VIII.72) nonetheless showed the highly desirable property of being active against MTX-resistant L1210 leukaemia. Thus, while treatment with up to 9 mg/kg (q2d × 4) of MTX produced no increase in lifespan at all in mice inoculated with L1210/MTX cells, treatment with 3 mg/kg (q2d × 5) of (VIII.72) prolonged survival by 86%. Compound (VIII.71) (5-Me) was also somewhat active against L1210/MTX leukaemia, but not to the same degree. Against another L1210 subline resistant to 6-mercaptopurine (6MP) by virtue of a known defect in *de novo* purine synthesis at the level of HGPRTase, compounds (VIII.70)–(VIII.72) exhibited activity substantially greater than their activity against the parental tumour. In this system, (VIII.70) produced a > 285% ILS (all mice surviving at least 30 days) at 6 mg/kg (q2d × 10) and compared favourably with MTX, which afforded a 250% ILS at 0.75 mg/kg (q2d × 8) but gave fewer long-term survivors. Compound (VIII.72) at 9 mg/kg (q2d × 10) gave a > 275% ILS, while (VIII.71) at 1.5 mg/kg (q2d × 10), its maximally tolerated dose in this system, gave only a 225% ILS. Another finding in this study which was potentially of practical importance in this study was that, while resistance to (VIII.70) could be induced *in vivo*, this required more time to bring about than resistance to MTX. Given the major therapeutic limitations imposed by acquired MTX resistance, this property seemed to augur well for further development of the quinazoline aspartates as anticancer drugs.

As part of the preclinical studies on these agents, a number of studies aimed at selecting the most promising member of the series for eventual clinical trial were carried out [48]. *In vitro* growth inhibition assays were performed with additional L1210 murine leukaemia cell lines with growth requirements somewhat different from those used earlier, and this time it was found that the

5-substituted derivatives, with IC_{50} values of 6–8 nM, were actually more potent than the compound without a 5-substituent, whose IC_{50} value was roughly 3-fold higher. Moreover, against these cell lines, the quinazolines were more nearly equipotent with MTX than had previously been observed. Toxicities were also determined in normal mice, and it was found that, while (VIII.71) and (VIII.72) had about the same LD_{50} value as MTX (3–4 mg/kg, qd × 5), the 5-methyl derivative (methasquin, (VIII.70)) on the same schedule was approximately 10-times more toxic (LD_{50} = 0.4 mg/kg). This was also true when single doses were given, with (VIII.70) giving an LD_{50} of 47 mg/kg as compared with 92 mg/kg for MTX, and 170–190 mg/kg for (VIII.71) and (VIII.72). Thus, methasquin appeared to have a narrower margin of safety than quinaspar or chlorasquin. Moreover, the toxicity of methasquin was less readily reversed with leucovorin than was the toxicity of MTX, and the optimal timing of administration of leucovorin was different for the two drugs. For example, simultaneous treatment of L1210 leukaemic mice on a qd × 5 schedule with 12 mg/kg of MTX and 20 mg/kg of leucovorin resulted in complete abrogation of both antitumour effect and host toxicity (as measured by weight loss), whereas 12 mg/kg of MTX followed 16 h later by 20 mg/kg of leucovorin produced a 175% ILS and still prevented weight loss. In contrast, treatment of the mice with 3 mg/kg of methasquin and 20 mg/kg of leucovorin, either simultaneously or with a 16 h delay, produced a 75% ILS but also gave considerable weight loss (*ca.* 20%) consistent with non-reversible host toxicity. It was speculated that the poorer ability of leucovorin to protect host cells from the toxic action of methasquin in comparison with MTX reflected tighter binding to DHFR, as subsequently demonstrated by Albrecht *et al.* [268] with enzyme from Chinese hamster cell lines. However, in an apparent reference to other laboratory studies already under way at the Sloan-Kettering Institute, it was also suggested that 'different kinds of *in vivo* association' might be occurring.

As mentioned above, the quinazoline aspartates are potent inhibitors of DHFR, and in fact, may be more tightly bound to the enzyme than either MTX or AMT. Albrecht *et al.* [268] observed essentially stoichiometric inhibition of DHFR's from two lines of Chinese hamster cells, and also noted that, depending on the enzyme studied, dihydrofolate was much less successful in reversing the inhibitory effect of methasquin than of MTX or AMT. This was especially true when the dose-response for inhibition of enzyme activity by MTX and AMT displayed a curvilinear pattern indicative of a relatively high 'off-rate'. Since this property was perceived as a likely basis for acquired antifolate resistance via enzyme mutation, the quinazoline aspartates were seen as possessing a distinct advantage over MTX and AMT.

That the differences observed *in vivo* between methasquin and the classical antifolates MTX and AMT were not due solely to the more nearly stoichiometric interaction of the quinazoline with DHFR was revealed in a paper published in 1972 by Sirotnak and Donsbach [269]. A sensitive method was developed to study the kinetics of transport of non-radioactive antifolates by assaying for residual DHFR activity in cell lysates at different times after exposure to the drug. Using this technique, the authors determined that the K_m for transport of methasquin into L1210 cells was 27 μM, whereas for MTX and AMT this value was 4.4 and 1.4 μM, respectively. The quinazoline aspartate therefore had a lower affinity than MTX for the carrier protein responsible for the active transport of MTX and reduced folates, and its V_{max} was approximately 2-fold lower as well. Methasquin uptake was competitively inhibited by adding either MTX or AMT to the medium, demonstrating that the quinazoline shared a common transport pathway with the pteridines. It should be noted, however, that the kinetics of methasquin uptake were more complex than those of MTX uptake, and that, in particular, the quinazoline exhibited a very high initial uptake component followed by a slower second phase, whereas the initial uptake of the pteridines was slower but eventually produced comparable intracellular levels of drug. In light of what is known today, this difference in cellular pharmacokinetics presumably reflects intracellular polyglutamylation, which does not occur when the side-chain is aspartic instead of glutamic acid. Most importantly, the rate of efflux of methasquin from cells was very slow in comparison with that of MTX or AMT. As a result, there remained in the cells a considerable amount of drug in excess of the amount needed to just saturate the DHFR. Since a high intracellular concentration of non-bound drug would be expected to minimize competition by dihydrofolate which accumulates in the cell during DHFR inhibition, the authors suggested that the *in vivo* activity of methasquin was due in part to the fact that free drug was retained efficiently in tumour cells, even in the face of a rapid decline in systemic levels due to renal or hepatobiliary clearance.

An important study on the inhibition of DHFR activity in mouse small intestine by methasquin was carried out with the aim of determining whether differences observed *in vivo* between this drug and MTX might be reflective of differences in enzyme inhibition in this most sensitive mouse tissue [270]. A pivotal conclusion of this study was that the *duration* of inhibition of DHFR was the single factor that contributed most strongly to toxicity. Support for this idea came from the finding that the relative potencies of methasquin and MTX as inhibitors of DNA synthesis in mouse small intestine did not correlate with the lethality of the two drugs. The qualitative results in intestinal

cells were similar to those in L1210 cells, in that methasquin was taken up more slowly than MTX and was retained for longer periods. Thus, a 30 mg/kg dose of MTX had to be given in order to inhibit DNA synthesis in intestinal crypt cells by 50%, whereas with methasquin the same effect was achieved with 0.4 mg/kg. This is rather remarkable, given the fact that methasquin is not an especially lipophilic molecule and is not known to form polyglutamates, both of which would tend to increase retention. In light of what is known today about the mechanisms by which antifolates leave the cell [271], it would appear that the high retention of methasquin in comparison with MTX may be due to decreased affinity for an efflux carrier.

Sirotnak and Donsbach [272] have performed a careful analysis of the relationship between the chemical structure of an antifolate and its uptake into cells. Among the compounds in this study were the quinazoline aspartates (VIII.70)–(III.72). The K_m for influx was determined to be 38 μM for (VIII.70), 27 μM for (VIII.71), and 24 μM for (VIII.72), while the rate constants for efflux into drug free medium were essentially identical ($k = 0.017–0.019\ min^{-1}$). Since the K_m for MTX transport was 5 μM and the rate constant for efflux was 0.020 min^{-1}, it might be concluded at first glance that MTX would be taken up more effectively. However, when the ratios of free intracellular drug to extracellular drug were calculated (all at the same extracellular concentration of 2.2 μM), they were found to be 5.4 for (VIII.70) (quinaspar), 8.8 for (III.71) (methasquin), and 10.2 for (VIII.72) (chlorasquin). The ratio for MTX was 3.4 and that for AMT was 5.1. Thus, methasquin and especially chlorasquin were superior to MTX and AMT with regard to their ability to accumulate in cells. When the same ratios were calculated for the corresponding quinazoline glutamates, on the other hand, they were all found to exceed the ratios for the aspartates. As a result, cellular accumulation of free drug exceeded the DHFR level by an even greater amount, resulting in a marked increase in toxicity. It appeared from these results that there probably existed a greater degree of selectivity between tumour and normal cells with respect to the retention of quinazoline aspartates than quinazoline glutamates. Stated differently, even though aspartates were less potent than glutamates, they were very likely to be more therapeutically selective.

An unusual observation in this transport study [272] was that the D-enantiomer of quinaspar ((VIII.70)) was taken up very efficiently, with a K_m value of 7.7 μM as compared with 5.0 μM for MTX and 38 μM for L-(VIII.70). The ratio of free intracellular drug to external drug was 8.9 for D-(VIII.70) as compared to 5.4 for L-(VIII.70). However, the rate constant for efflux was 3-fold higher for D-(VIII.70) than for L-(VIII.70). It may be recalled that, in cell growth inhibition assays [51], the two enantiomers had proved to be

nearly equipotent. The fact that D-(VIII.70) influx was faster than L-(VIII.70) influx while, at the same time, D-(VIII.70) efflux was faster than L-(VIII.70) efflux was most interesting because it supported the notion that influx and efflux carrier proteins for antifolates may have somewhat different binding requirements for their substrates.

In addition to their other studies on methasquin, Sirotnak and Donsbach [273] also put this agent to good use as a probe of the selectivity of response of murine tumours to antifolate therapy. Their analysis brought together in a single elegant pharmacologic model many of the concepts that had been developed earlier. Mice were inoculated i.p. with L1210 leukaemia, and, after treatment with equal doses (3 mg/kg) of MTX, AMT, methasquin, or 5-chloro-5,8-dideazaAMT, the extent and duration of DNA synthesis inhibition in the tumour cells and in cells of the intestinal epithelium were compared. The peak level of free methasquin and 5-chloro-5,8-dideazaAMT in L1210 cell was 5- to 8-fold in excess of the DHFR level by 1–2 h, whereas this level for MTX and AMT was only half as high. At 16 h, the level of non-bound quinazolines still exceeded that of DHFR, while the level of nonbound MTX and AMT had declined to that of the enzyme. The levels of free methasquin and 5-chloro-5,8-dideazaAMT followed a similar pattern in cells of the small intestine, though absolute amounts of drug expressed per wet weight of tissue were lower. On the other hand, AMT levels in intestinal cells were like those of methasquin and 5-chloro-5,8-dideazaAMT, rather than those of MTX, and were consistent with the known high toxicity of AMT in mice. When drug levels were compared not at equal doses, but at maximally tolerated doses (0.01 mg/kg for 5-chloro-5,8-dideazaAMT, 0.3 mg/kg for AMT, 0.75 mg/kg for methasquin), the results were striking. Under these circumstances the ratio of free drug to DHFR in L1210 cells followed the order methasquin (20-fold) > MTX (13-fold) > AMT (6-fold) > 5-chloro-5,8-dideazaAMT (4-fold), whereas in intestinal cells at the maximally tolerated dose the ratio of free drug to DHFR was 8-fold for MTX and < 2-fold for the other drugs. When DNA synthesis inhibition was determined by measuring [^{3}H]uridine incorporation in the leukaemia cells and the intestinal cells, the duration of 50% inhibition was found to be twice as long in the leukaemic cells as in the intestinal cells, and followed the order methasquin = MTX > AMT > 5-chloro-5,8-dideaza-AMT. While there appeared to be a small discrepancy between the duration of DNA synthesis by methasquin and MTX on the one hand, and the relative cellular accumulation of the two drugs on the other, the results suggested clearly that, among the four drugs, methasquin would be the most therapeutically selective, and 5-chloro-5,8-dideazaAMT the least. In the main, the results of this analysis were in excellent agreement with the *in vivo* data

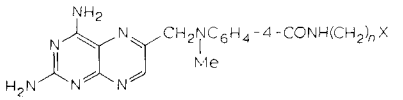
obtained earlier [48, 51] and argued for methasquin as the most promising of the quinazoline aspartate analogues.

A Phase I clinical trial was carried out with methasquin and the outcome was briefly reported in 1972 [274]. The effects of the drug in humans were found to be, in general, similar to those of AMT. In view of the enormous clinical experience which had, by then, accrued with MTX, it was decided that the pharmacological rationale underlying the development of methasquin was not compelling enough to justify further work. For all practical purposes, MTX became the DHFR inhibitor of choice for anticancer treatment and methasquin was put on the shelf while efforts were launched to find antifolates targeted toward other enzymes. This critical change in research focus was really a watershed decision, and prompted medicinal chemists to begin an intensive search for inhibitors of (i) thymidylate synthase and (ii) the purine biosynthetic enzymes (for example, the GAR and AICAR transformylases). As documented elsewhere in this review, some potent new weapons were added to the antifolate armamentarium as a result of this effort, including the thymidylate synthase inhibitor CB3717 and the purine nucleotide biosynthesis inhibitor 5,10-dideaza-5,6,7,8-tetrahydrofolate. In this sense, therefore, the work done on quinazoline aspartates constituted an important chapter in the history of antifolates.

AMINO MONOSULPHONIC AND AMINO MONOPHOSPHONIC ACID ANALOGUES

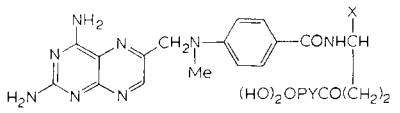
Several MTX analogues in which the glutamate moiety was replaced by ω-aminoalkanesulphonic and ω-aminoalkanephosphonic acids containing up to five CH_2 groups were synthesized recently [275] as part of a larger study examining the structural requirements for binding to folylpolyglutamate synthetase (FPGS) by folates and antifolates. Silylated aminomethanesulphonic, 2-aminoethanesulphonic, 3-aminopropanesulphonic, and 4-aminobutanesulphonic acids were condensed with 4-amino-4-deoxy-N^{10}-methylpteroic acid (MeAPA) in the presence of diethyl phosphorocyanidate (DEPC) to obtain the sulphonic acids (VIII.74)–(VIII.77) in 35–70% yield depending on the length of the $(CH_2)_n$ chain. Condensation of MeAPA with diethyl 3-aminopropanephosphonate in the presence of DEPC afforded the diethyl ester (VIII.78) (60% yield), which on treatment with Me_3SiBr at room temperature for 6 days, followed by quenching with MeOH, formed a complex mixture of products, two of which were the monoethyl ester, (VIII.79), and the free acid, (VIII.80). Monoester (VIII.79) could also be prepared from monoethyl 3-aminopropanephosphonate by the DEPC method. Acid (VIII.80) was also

obtained by treating diethyl 3-aminopropanephosphonate with Me_3SiBr and directly adding the crude product, which was assumed to be a bis(trimethylsilyl)phosphonate ester, directly to DEPC-activated MeAPA. A similar process starting from diethyl 2-aminoethanephosphonate yielded acid (VIII.81). With the exception of the synthesis of diester (VIII.78), yields in all these reactions were poor. In assays against FPGS from mouse liver, compounds (VIII.74)–(VIII.77) and (VIII.79)–(VIII.81) proved to be only weak inhibitors (< 30% inhibition at equal substrate and inhibitor concentration), and showed no variation in activity as a function of chain length. The very low activity of these compounds confirmed that the α-carboxyl group plays a critical role in the binding of inhibitors to the active site of FPGS and should be retained in designing such inhibitors.

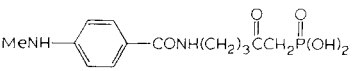


(VIII.74) n = 1, X = SO_2OH (VIII.78) n = 3, X = $PO(OEt)_2$
(VIII.75) n = 2, X = SO_2OH (VIII.79) n = 3, X = $PO(OH)OEt$
(VIII.76) n = 3, X = SO_2OH (VIII.80) n = 3, X = $PO(OH)_2$
(VIII.77) n = 4, X = SO_2OH (VIII.81) n = 2, X = $PO(OH)_2$

An interesting approach to inhibitors of FPGS has involved analogues of the acyl phosphate (VIII.82), which has been strongly implicated [276] as the likely active intermediate in the glutamylation reaction. In one example of this approach, Tang and Coward [277] attempted to synthesize the phosphonic acid analogue (VIII.83), which bears an obvious structural resemblance to (VIII.82). Although they did not succeed in this task, they did prepare the model compound (VIII.84) from 2,4-diamino-6-bromomethylpteridine and *N*-[4-(*N*-methylamino)benzoyl]-5-amino-2-oxopentanephosphonic acid (VIII.85). Compound (VIII.85) was prepared from methyl 4-chlorobutyrate by a multistep sequence consisting of (i) reaction with NaN_3, (ii) condensation with dimethyl methanephosphonate and *n*-BuLi, (iii) conversion of the keto group in the resulting β-ketophosphonate ester to an ethyleneketal, (iv) catalytic reduction of the azido group to an amine and direct conversion to an *N*-(4-nitrobenzoyl) derivative, (v) reduction of the nitro group, (vi) con-



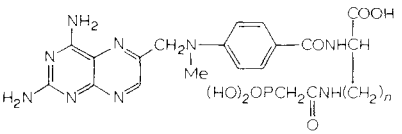
(VIII.82) X = COOH, Y = O
(VIII.83) X = COOH, Y = CH_2
(VIII.84) X = H, Y = CH_2



(VIII.85)

densation of the resultant amine with 4-(*N*-Cbz-amino)benzoyl chloride; (vii) removal of the Cbz group by hydrogenation; and (viii) cleavage of the ethylene ketal and phosphonate ester groups with Me_3SiBr. The yield of (VIII.85) from the ketal diester was estimated to be 20%, but the overall yield in this lengthy scheme was less than 1%.

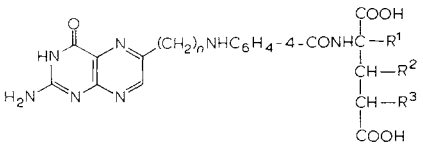
A structure somewhat related to the acyl phosphonate analogue (VIII.83) has been briefly described [278] in a report on FPGS inhibitors. The preparation of this compound (VIII.86) was achieved from N^{α}-(4-amino-4-deoxy-N^{10}-methylpteroyl)-L-lysine and 4-nitrophenyl phosphonoacetate. While (VIII.86) obviously contained too many CH_2 groups to be considered a close analogue of (VIII.83), the shorter-chain compound (VIII.87), a derivative of N^{α}-(4-amino-4-deoxy-N^{10}-methylpteroyl)-L-2,3-diaminobutanoic acid, would seem to be reasonable candidate for this role.



(VIII.86) $n = 4$
(VIII.87) $n = 1$

α-, β- AND γ-SUBSTITUTED GLUTAMIC ACID ANALOGUES

The largest number of compounds of this type in any single series was reported in 1967 by Plante *et al.* [72], who replaced the L-glutamic acid moiety in folic and homofolic acid by DL-α-, DL/DL-β-, or DL/DL-γ-methylglutamic acid to obtain the analogues (VIII.88)–(VIII.93), and by DL/DL-γ-hydroxyglutamic and DL/DL-γ-aminoglutamic acid to obtain the analogues (VIII.94)–(VIII.97). All these compounds were prepared from the 2-acetyl-N^{10}-trifluoroacetyl derivatives of pteroic and homopteroic acid and the diethyl esters of the amino acids via mixed anhydride coupling followed by simultaneous hydrolysis of ester and amide groups with base (0.1 M NaOH, 100 °C for 20–30 min under nitrogen). Yields were generally less than 20%, but enough of each compound was obtained for biochemical studies. Reduction (VIII.88)–(VIII.97) (all as mixtures of diastereomers) with sodium dithionite afforded the corresponding 7,8-dihydro compounds, which on further reduction with DHFR were converted to 5,6,7,8-tetrahydro derivatives with C_6 in the 'natural' configuration. It may be noted that γ-hydroxyfolic acid (VIII.94), as a mixture of diastereomers, has been prepared from *N*-(4-aminobenzoyl)-γ-hydroxyglutamic acid, 2,4,5-triamino-6(5*H*)-pyrimidinone, and 2,3-dibromopropionaldehyde [271].



(VIII.88) $n = 1$, $R^1 = Me$, $R^2 = R^3 = H$
(VIII.89) $n = 1$, $R^1 = R^3 = H$, $R^2 = Me$
(VIII.90) $n = 1$, $R^1 = R^2 = H$, $R^3 = Me$
(VIII.91) $n = 2$, $R^1 = Me$, $R^2 = R^3 = H$
(VIII.92) $n = 2$, $R^1 = R^3 = H$, $R^2 = Me$
(VIII.93) $n = 2$, $R^1 = R^2 = H$, $R^3 = Me$
(VIII.94) $n = 1$, $R^1 = R^2 = H$, $R^3 = OH$
(VIII.95) $n = 1$, $R^1 = R^2 = H$, $R^3 = NH_2$
(VIII.96) $n = 2$, $R^1 = R^2 = H$, $R^3 = OH$
(VIII.97) $n = 2$, $R^1 = R^2 = H$, $R^3 = NH_2$

Substitution of the α, β- and γ-carbons in the glutamate side-chain of folic acid and homofolic acids was remarkably well tolerated by dihydrofolate reductase in both the folate and homofolate series, irrespective of the fact that the substrates used in the reaction were always mixed diastereomers [72]. The relative rates of reduction of H_2-(VIII.88), H_2-(VIII.89), and H_2-(VIII.92) were approximately the same as those of dihydrofolate and dihydrohomofolate, while the relative rates of reduction of sterically more hindered α-methyl analogues H_2-(VIII.88) and H_2-(VIII.91) were decreased only 2- to 3-fold. Reduction of the H_2-(VIII.94)–(VIII.97) proceeded at a relative rate 2- to 3-fold lower than that of the γ-methyl analogues H_2-(VIII.90) and H_2-(VIII.93), suggesting that polar substituents at the γ-position are less well tolerated in the active site than a small hydrophobic alkyl group. Unfortunately, kinetic analyses were not performed in this study, and thus it is not known whether these observed differences in relative rates were the result of a higher K_m or a lower V_{max}. It should also be noted that relatively high tolerance for α-, β- and γ-substitution in folic and homofolic acids does not necessarily mean that the same structure modifications would be equally tolerated in MTX and other 2,4-diaminopteridine antifolates, since the latter are known to bind to DHFR in a different orientation from dihydrofolate [229].

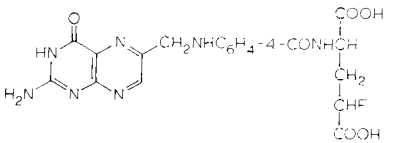
The ability of the α-, β- and γ-substituted tetrahydrohomofolate analogues H_4-(VIII.91)–H_4-(VIII.93), H_4-(VIII.96), and H_4-(VIII.97) to serve as substrates for *E. coli* thymidylate synthase (TS) was measured [72], and it was found that this enzyme is more sensitive than DHFR to substitution in the glutamate moiety. Neither H_4-(VIII.91) (α-methyl) nor H_4-(VIII.97) (γ-amino) functioned as a substrate, while the reaction rates of H_4-(VIII.92) (β-methyl), H_4-(VIII.93) (γ-methyl), and H_4-(VIII.96) (γ-hydroxy) relative to tetrahydro-

homofolate were reduced 2-, 3- and 5-fold, respectively. It appeared from these results that TS binding was sensitive to substitution next to either the α- or γ-carboxyl group, and that polar substitution (especially with a positively charged amino group) next to the γ-carboxyl was less well tolerated than substitution with a small hydrophobic alkyl group. These effects were also reflected accurately in the abilities of H_4-(VIII.91)–H_4-(VIII.93), H_4-(VIII.96), and H_4-(VIII.97) to competitively inhibit TS.

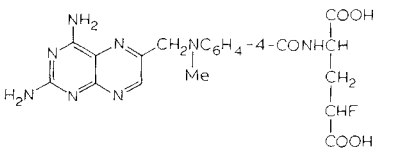
In a kinetic analysis of the cofactor activity of the tetrahydrofolate analogues H_4-(VIII.90) (γ-methyl), the K_m was found to be 2-fold higher than that of tetrahydrofolate, while the V_{max} was 2-fold lower. In a similar analysis of the activity of H_4-(VIII.95) (γ-hydroxy), K_m remained unchanged while V_{max} decreased 8-fold. Substitution thus seemed to have a different effect on K_m and V_{max} depending on the nature and/or location of the substituent.

The ability of the α-methyl derivatives (VIII.89) and H_4-(VIII.89) to act as either substrates of inhibitors of hog liver FPGS was recently examined [246]. The aromatic analogue was inactive below 5 mM when (6*S*)-tetrahydrofolate was used as the substrate, while the reduced compound was inactive below 100 μM. Neither compound was a substrate. Thus, a methyl group next to the α-carboxyl very profoundly impaired the ability of the folate molecule to bind to the active site of this enzyme. This was in remarkable contrast to the effect of the α-methyl substituent on the activity of H_2-(VIII.88) as a substrate for DHFR.

Compound (VIII.98), a folic acid analogue with a γ-fluoro substituent in the side-chain was described first by Alekseeva *et al.* [281] and several years later by Bergmann and Chun-Hsu [282]. The synthesis of (VIII.98), as mixture of *threo* and *erythro* isomers, was achieved via the Waller method from *N*-(4-aminobenzoyl)-γ-fluoroglutamic acid, 2,4,5-triamino-6(1*H*)-pyrimidinone, and 2,3-dibromopropionaldehyde, but the yield was low (5.6%). γ-Fluoroglutamic acid, as a mixture of D- and L-enantiomers, was prepared from diethyl 2-fluoromalonate by condensation with ethyl 2-acetamidoacrylate followed by hydrolysis and decarboxylation in refluxing 12 M HCl, or from ethyl 3-chloro-2-hydroxypropanoate by a sequence consisting of (i) *O*-*t*-butylation with $CH_2=C(CH_3)_2$, (ii) condensation with diethyl 2-acetamidomalonate, (iii)



(VIII 98)



(VIII 99)

cleavage of the *O*-*t*-butyl group with *p*-TsOH; (iv) fluorination with *N*(2-chloro-1,1,2-trifluoroethyl)-*N*,*N*-diethylamine; and (v) hydrolysis and decarboxylation in boiling 12 M HCl. Further reaction of γ-fluoroglutamic acid with *N*-hydroxysuccinimidoyl 4-(*N*-Cbz-amino)benzoate followed by removal of the Cbz group with HBr afforded the *N*-(4-aminobenzoyl)amino-γ-fluoroglutamic acid required for the Waller reaction.

More recently Galivan *et al.* [283] described a synthesis of γ-fluoroMTX (VIII.99) involving condensation of di-*t*-butyl *N*-[4-(*N*-methylamino)benzoyl]-γ-fluoro-L-glutamate with 2,4-diamino-6-bromomethylpteridine, followed by hydrolysis of the ester groups with trifluoroacetic acid. The overall yield was 45%, and two products with *erythro* and *threo* stereochemistry were shown to be present in equal amounts by ^{19}F-NMR and ion-exchange HPLC. The proton NMR spectrum of the mixture, taken in D_2O-DCl solution, showed the β-CH_2 protons as a multiplet at δ 2.94 and the γ-CHF proton as a markedly deshielded multiplet at δ 5.42.

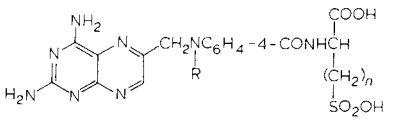
Assays of DHFR inhibition by the mixed *erythro* and *threo* isomers of γ-fluoroMTX were carried out with enzymes from MTX-resistant *L. casei* (IC_{50} = 0.45 nM vs. 0.40 nM for MTX), human leukaemic spleen (IC_{50} = 0.95 nM vs. 0.75 nM for MTX), and H35 rat hepatoma cells (IC_{50} = 0.96 nM vs. 0.88 nM for MTX) [283]. Thus, introduction of a γ-fluoro substitution had essentially no effect on binding to DHFR. A comparative study of the uptake of MTX and γ-fluoroMTX by confluent and dividing H35 cells was also carried out, using DHFR titration to determine intracellular drug levels. In confluent cells, the initial rate of uptake of MTX was somewhat more rapid than that of γ-fluoroMTX, but the overall accumulation of the two drugs over 2 h was approximately the same, reflecting the fact that MTX polyglutamylation is not extensive under these conditions. By contrast, whereas drug uptake in cells treated with MTX increased steadily over 2 h, reflecting intracellular polyglutamylation of MTX, γ-fluoroMTX reached a steady-state level in about 30 min, as expected from a non-polyglutamylated compound. As a result of this difference, the total MTX pool (that is, MTX plus MTX polyglutamates) after 2 h was 5- to 6-fold greater than that γ-fluoroMTX. Efflux experiments similarly showed that cells pre-loaded with MTX and then placed in drug-free medium lost drug more slowly than cells treated identically with γ-fluoroMTX. Inefficient γ-glutamylation of γ-fluoroMTX was demonstrated directly in an *in vitro* assay using partially purified FPGS from rat liver. The substrate activity of γ-fluoroMTX was estimated to be only 5% of that of MTX, and in fact γ-fluoroMTX could be shown to be an inhibitor of MTX polyglutamylation. Its inhibitory potency was quite low, however, as evidenced by the fact that when 1 μM MTX and

100 μM γ-fluoroMTX were added to the enzyme, conjugation of L-[^{3}H]glutamate to MTX was reduced by only 20% relative to the control value with MTX alone. As expected from the fact that γ-fluoroMTX was poorly glutamylated relative to MTX, the two drugs showed markedly different effects on cell growth depending on length of treatment. For example, while the IC_{50} values of γ-fluoroMTX and MTX for an exposure time of 2 h were 700 and 0.3 μM, respectively, the corresponding values for 72 h were 0.12 and 0.01 μM. Thus there was a 2300-fold difference in potency between the two drugs for the short treatment, but only a 12-fold difference for the longer exposure. Viewed differently, the data showed that changing the length of treatment from 72 to 2 h increased the IC_{50} value of MTX only 30-fold, but increased that of γ-fluoroMTX 6000-fold. That the divergent effects of the two drugs on cell growth as a function of the duration of treatment reflected differences in dTMP synthesis was unequivocally demonstrated by the results of a tritium release experiment using [5-^{3}H]deoxyuridine. For example, treatment of H35 cells with 5 μM MTX for 2 h resulted in complete inhibition of dTMP synthesis for up to 24 h as measured by tritiated water release into the medium, while the same treatment with 300 μM γ-fluoroMTX (a 60-fold higher concentration) led to complete inhibition for only 2 h.

REPLACEMENT OF γ-CARBOXYL BY OTHER ACIDIC GROUPS

The effect of substituting other acidic groups for carboxyl at the γ-position of folates and antifolates has been of interest in recent years because of the possibility that these compounds would inhibit not only DHFR but also FPGS [284–287].

γ-Sulphonic acid analogues of MTX as well as AMT were prepared and studied in a variety of test systems by Rosowsky *et al.* [284, 285]. Condensation of 4-amino-4-deoxy-N^{10}-methylpteroic acid (MeAPA) with trimethylsilylated DL-homocysteic acid in the presence of diethyl phosphorocyanidate afforded the MTX analogue DL-VIII.100 [284]. Subsequently MeAPA was



(VIII.100) R = Me, n = 2 (VIII.102) R = H, n = 2
(VIII.101) R = Me, n = 1 (VIII.103) R = H, n = 1

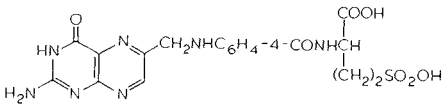
also elaborated via the same procedure to L-VIII.100 (77% yield) and to the L-cysteic acid derivative L-(VIII.101) (79% yield) [285]. In addition, 4-amino-4-deoxy-N^{10}-formylpteroic acid was converted to a mixed anhydride, condensed with L-homocysteic and L-cysteic acids, and deprotected with a minimum amount of NaOH at room temperature to obtain the AMT analogues L-VIII.102 (83%) and L-(VIII.103) (72%), respectively. All four compounds were isolated as partial ammonium salts after purification by ion-exchange chromatography with NH_4HCO_3 as the eluting buffer.

The activity of the sulphonic acids (VIII.100)–(VIII.103) as DHFR inhibitors has been reported to be very similar to that of MTX and AMT [284, 285]. The IC_{50} values of DL-(VIII.100) and MTX as inhibitors of DHFR from L1210 cells (competitive [^{3}H]MTX binding assay) were both found to be 1.0 nM, while the IC_{50} value of DL-(VIII.100) against enzyme from *L. casei* was 17 nM as compared with 10 nM for MTX [284]. In subsequent experiments on the products obtained from L-homocysteic and L-cysteic acids [285], compounds L-(VIII.100)–L-(VIII.103) were found to have the following IC_{50} values against DHFR from L1210 cells (spectrophotometric assay): L-(VIII.100), 41 nM; L-(VIII.101), 45 nM; L-(VIII.102), 63 nM; L-(VIII.103), 65 nM. The IC_{50} values of MTX and AMT in this assay were 50 and 40 nM, respectively. Thus, replacement of the γ-COOH group in MTX and AMT by γ-SO_2OH led to a less than 2-fold loss of affinity as measured by the IC_{50}.

Investigation of the interaction of the sulphonic acids DL-(VIII.100) and L-(VIII.100)–L-(VIII.103) with FPGS from mouse liver revealed these compounds to be competitive inhibitors, with K_i values of $188 \pm 56\,\mu$M (DL-(VIII.100)), $198 \pm 31\,\mu$M (L-(VIII.100)), $136 \pm 31\,\mu$M (L-(VIII.101)), $59 \pm 28\,\mu$M (L-(VIII.102)), and $43 \pm 8\,\mu$M (L-(VIII.103)). Three conclusions made on the basis of these results were (i) that inhibition of FPGS was similar with DL-(VIII.100) and L-(VIII.100), (ii) that AMT analogues were more active than MTX analogues of the same chain length, and (iii) that the homocysteic acid analogues ($n = 2$) were more active than the cysteic acid analogues ($n = 1$). The most potent inhibitor in this group (L-VIII.103) had a K_i approximately 4-fold lower than the K_m of folic acid or MTX as substrates. Studies on the inhibition of partially purified FPGS from human liver by the sulphonic acid analogues of MTX and AMT have also been reported [288]. The L-homocysteic acid analogues L-(VIII.100) and L-(VIII.102) had estimated K_i values of 131 ± 5 and $35 \pm 2\,\mu$M, respectively, whereas the corresponding values for the L-cysteic acid analogues L-(VIII.101) and L-(VIII.103) were 173 ± 6 and $66 \pm 5\,\mu$M. Interestingly, the kinetics of inhibition observed with the human enzyme were strictly competitive for L-(VIII.100) and L-(VIII.102), but were of the mixed type for L-(VIII.101) and L-(VIII.103). While the latter

results were consistent with binding to a second site, the nature of this interaction remains unclear.

The results with L- and DL-(VIII.102) may be compared with data recently reported for the corresponding folate analogue (VIII.104) and the reduced derivatives H_2-(VIII.104) and H_4-(VIII.104) (all in the DL form) [246], whose K_i values, obtained by adding a fixed concentration of inhibitor to various concentrations of folate or (6*S*)-tetrahydrofolate as the substrate, were estimated to be 278, 28 and 43 μM, respectively. The fact that the reduced derivatives were approximately 10-fold better inhibitors than the parent compound was consistent with the greater substrate efficiencies of reduced folates relative to folate, and suggested that a reduced B ring might be a desirable feature in FPGS inhibitors. It should be noted, however, that there is, as yet, no evidence that this is true for 2,4-diamino as well as 2-amino-4(3*H*)-oxo derivatives.



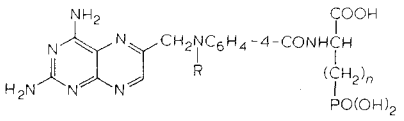
(VIII.104)

In vitro cell growth inhibition assays using L1210 murine leukaemia cells revealed that the activities of the sulphonic acid analogues of MTX and AMT were in qualitative agreement with their enzyme inhibitory activities, with IC_{50} values of 0.18 μM (L-(VIII.100)), 0.34 μM (L-(VIII.101)), 0.031 μM (L-(VIII.102), and 0.44 μM (L=(VIII.103)), as compared with 0.012 μM for MTX and 0.0031 μM for AMT [285]. An interesting aspect of these results was that the sulphonic acid analogues of AMT were more active than those of MTX as cell growth inhibitors even though the reverse was true where DHFR inhibition was concerned. This suggested either that the AMT analogues were taken up more efficiently into the cells, or that these compounds were inhibiting not just DHFR but also a second biochemical locus, which was postulated to be FPGS. Dual inhibition of these enzymes has been suggested as a possible mechanism of 'self-potentiation' [284]. According to this concept, interference with the polyglutamylation of reduced folates would enhance the ability of the DHFR inhibitor to arrest DNA synthesis, and hence block cell growth.

In vivo antitumour experiments with compounds L-(VIII.100)–L-(VIII.103) were performed against L1210 leukaemia in mice [285]. Interestingly, despite the small differences between the homocysteic and cysteic acid analogues

in vitro, the former were substantially more effective *in vivo*. The MTX analogue L-(VIII.100) produced a 144% ILS at a dose of 32 mg/kg (bid × 10), while the AMT analogue L-(VIII.102) on the same schedule produced a 138% ILS at 2 mg/kg and a 100% ILS at 1 mg/kg. By comparison, 1 mg/kg of MTX gave a 133% ILS and 0.24 mg/kg of AMT gave a 138% ILS. Thus, the AMT analogue L-(VIII.102), when administered on a frequent schedule, was nearly equal to MTX both in potency and therapeutic effect. This compound was therefore the first example of a *classical* antifolate with substantial *in vivo* activity, despite the fact that it cannot form polyglutamates.

The MTX and AMT analogues DL-(VIII.105) and DL-(VIII.106), in which the γ-COOH group is replaced by γ-$PO(OH)_2$, have likewise been investigated [286]. Methyl DL-2-amino-4-phosphonobutyrate was prepared from the parent acid with $SOCl_2$/MeOH, and was condensed with 4-amino-4-deoxy-N^{10}-methylpteroic acid by the diethyl phosphorocyanidate method and with 4-amino-4-deoxy-N^{10}-formylpteroic acid by the mixed anhydride method. Deprotection with base then gave DL-(VIII.105) (21%) and DL-(VIII.106) (52%), respectively. AMT analogues DL-(VIII.107)-DL-(VIII.109) were subsequently prepared from the benzyl esters of appropriate α-amino-ω-phosphonoalkanoic acids (n = 1,3,4) by mixed anhydride or nitrophenyl ester coupling to 4-amino-4-deoxy-N^{10}-formylpteroic acid followed by hydrolysis with base [285].



(VIII.105) R = Me, n = 2 (VIII.108) R = H, n = 3
(VIII.106) R = H, n = 2 (VIII.109) R = H, n = 4
(VIII.107) R = H, n = 1

In assays of DHFR inhibition using purified enzyme from L1210 enzyme, the MTX analogue DL-(VIII.105) and AMT analogue DL-(VIII.106) had IC_{50} values of 6.5 and 6.0 nM as compared with 5.5 and 6.2 nM for L-(VIII.100) and L-(VIII.102), and 4 nM for both MTX and AMT [286]. Thus, replacement of the γ-COOH group in MTX and AMT by γ-$PO(OH)_2$ was no more detrimental to DHFR binding than replacement by γ-SO_2OH, even though the phosphonic acid contains an extra negative charge at physiological pH. Moreover, N^{10}-methyl substitution in the phosphonic acid series, as in the sulphonic acid series, did not seem to markedly influence DHFR binding. When the potency of the other members of the series wasexamined [285],

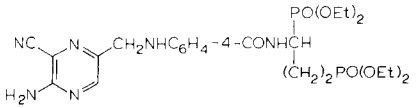
IC_{50} values of 0.55 μM (DL-(VIII.107)), 0.083 μM DL-(VIII.108)), and 0.067 μM (DL-(VIII.109)) were obtained. Thus, shortening the side-chain decreased DHFR binding, as with the aspartate analogue of MTX (*vide supra*), whereas addition of extra CH_2 groups had only a minor effect.

In assays of mouse liver FPGS inhibition *in vitro*, the MTX analogue DL-(VIII.105) had a K_i of 185 $\pm$ 71 μM, while the AMT analogue DL-(VIII.106) had a K_i of 8.4 $\pm$ 2.0 μM [285, 286, 289]. The other phosphonic acids were substantially less active, showing that the number of CH_2 groups in the side-chain is critical to the binding of inhibitors to the FPGS active site. Compound DL-(VIII.106) was approximately 5-fold more potent than the corresponding sulphonic acid, even though it was a DL-mixture, perhaps because the $PO(OH)_2$ group more closely mimics the acyl phosphonate intermediate in the glutamylation reaction. In assays against partially purified FPGS from human liver [288], DL-(VIII.105) and DL-(VIII.106) were determined to have K_i values of 83 $\pm$ 3 and 1.9 $\pm$ 0.1 μM, respectively. Thus, these phosphonic acid analogues appeared to be better inhibitors of the human than the mouse enzyme, suggesting that there probably exist some structural differences in the active sites of the two enzymes. Interestingly, while the kinetics of FPGS inhibition by the AMT analogue DL-(VIII.106) were strictly competitive, inhibition by the MTX analogue DL-(VIII.105) appeared to obey mixed kinetics consistent with binding to a second site. There was also a difference between the inhibition kinetics with the phosphonate DL-(VIII.105) and the sulphonate analogue L-(VIII.100) in that the former were of the mixed type while the latter were competitive. It is possible that this reflects binding of D-(VIII.105) to a second site, and that inhibition kinetics are observed with DL-(VIII.106) because D-(VIII.106) does not bind to this site. Further studies would be needed to address this point.

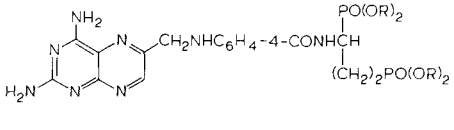
In cell culture assays against L1210 murine leukaemia cells, DL-(VIII.105) and DL-(VIII.106) had IC_{50} values of 0.19 and 0.035 μM, respectively [286]. These compounds were therefore approximately equipotent with the sulphonic acids and less potent than MTX or AMT, probably because they are less efficiently taken up by the cells and cannot form polyglutamates. *In vivo* experiments to compare the activity of the phosphonic acids against L1210 leukaemia were not carried out.

An interesting analogue of MTX containing a $PO(OH)_2$ group at both the α- and γ-position was sketchily described in 1984 by a French group [236, 290]. The *N*-benzylimine derivative of diethyl 3-phosphonopropionaldehyde was condensed with $HPO(OEt)_2$, and the product converted to a hydrochloride salt and reduced catalytically to obtain tetraethyl 1-amino-1,3-propanediphosphonate hydrochloride. The amino ester was acylated under

Schotten-Baumann conditions with 4-(*N*-Cbz-amino)benzoyl chloride, and the resulting amide was reduced catalytically and condensed with 2-amino-5-bromomethylpyrazine-3-carbonitrile to form the amino nitrile (VIII.110), which on annulation with guanidine yielded the ester (VIII.111). Treatment of (VIII.111) with Me_3SiBr followed by quenching with MeOH yielded the disphosphonic acid DL-(VIII.112). Antifolate activity was determined with bovine liver DHFR by spectrophotometric assay in the presence of excess enzyme. An IC_{50} value of 1.5 μM was obtained, as compared with 0.05 μM for MTX. Replacement of both COOH groups by $PO(OH)_2$ groups therefore led to a 300-fold decrease in binding. Since it was shown independently [286] that the MTX analogue with a $PO(OH)_2$ group only at the γ-position was almost as active as MTX, it appears that most of this loss of binding is due to the effect of replacing the α-COOH group by $PO(OH)_2$.



(VIII.110)



(VIII.111) R = Et
(VIII.112) R = H

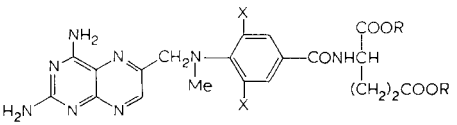
γ-CARBOXYL SUBSTITUTION WITH ESTER, AMIDE OR PEPTIDE GROUPS

This section includes not only MTX analogues in which the γ-COOH group is blocked as an ester, amide, or peptide, but also those in which both the γ- and α-COOH group is modified by addition of these groups. The corresponding α-substituted compounds are discussed elsewhere in this review (*vide supra*). It should be noted with regard to the diester derivatives that only compounds specifically made for biological testing purposes by esterification of preformed antifolates are discussed here. A multitude of diesters have also been described in the literature as intermediates in the synthesis of antifolates, and are cited in appropriate sections of this review. Temporary protection of the carboxyl groups in the amino-acid moiety during the synthesis and purification of antifolates has been achieved generally with methyl, ethyl, *i*-propyl, *t*-butyl or benzyl groups. In most instances, however, these diesters were directly converted to diacids and were not tested for biological activity.

γ-Monoesters and α.γ-diesters

Chemical synthesis

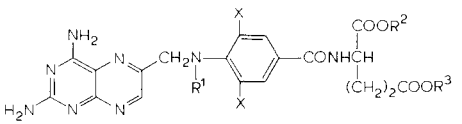
Although diesters of folates were sketchily described much earlier [291], the idea of using lipid soluble diesters of MTX as therapeutic agents, first proposed in 1962 [292], was not elaborated systematically until the early 1970's [293–298]. Direct esterification of MTX as well as DCM with a variety of primary and secondary aliphatic alcohols was shown to proceed in good yield with dry HCl [296] or boron trifluoride [293] as the catalyst, giving products (VIII.113)–(VIII.131), the lipophilicity of which could be shown to vary with the length of the alkyl group.



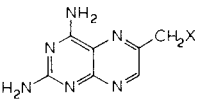
(VIII.113) X = H, R = Me
(VIII.114) X = H, R = Et
(VIII.115) X = H, R = *n*-Pr
(VIII.116) X = H, R = *i*-Pr
(VIII.117) X = H, R = *n*-Bu
(VIII.118) X = H, R = *i*-Bu
(VIII.119) X = H, R = *n*-Am
(VIII.120) X = H, R = *i*-Am
(VIII.121) X = H, R = 2-Am
(VIII.122) X = H, R = *n*-Hex
(VIII.123) X = H, R = *n*-Oct
(VIII.124) X = H, R = $EtOCH_2CH_2$
(VIII.125) X = Cl, R = Me
(VIII.126) X = Cl, R = Et
(VIII.127) X = Cl, R = *n*-Bu
(VIII.128) X = Cl, R = *i*-Bu
(VIII.129) X = Cl, R = *i*-Am
(VIII.130) X = Cl, R = 2-Am
(VIII.131) X = Cl, R = *n*-Oct
(VIII.132) X = H, R = $n\text{-}C_{12}H_{25}$
(VIII.133) X = H, R = $n\text{-}C_{16}H_{33}$
(VIII.134) X = H, R = $n\text{-}C_{18}H_{35}$
(VIII.135) X = H, R = *t*-Bu
(VIII.136) X = H, R = $CH_2C_6H_5$
(VIII.137) X = H, R = $CH_2C_6H_3(2,5\text{-}Me_2)$
(VIII.138) X = H, R = $CH_2C_6H_2(2,4,6\text{-}Me_3)$
(VIII.139) X = H, R = $CH_2C_6H_3(2,6\text{-}Cl_2)$
(VIII.140) X = H, R = CH_2(3-pyridyl)
(VIII.141) X = H, R = α-*n*-Bu-γ-CH_2(3-pyridyl)
(VIII.142) X = H, R = $CH_2C_6H_2$[6-Cl-3,4-(OCH_2O)]

More recently, Rosowsky and Yu [296, 297] obtained the MTX dialkyl and diaralkyl esters (VIII.132)–(VIII.142) by reaction of the parent acids with alkyl or aralkyl halides in DMSO in the presence of Cs_2CO_3. Yields varied with the reactivity of the alkylating reagent, and ranged from 30 to 75% except for the reaction of *t*-butyl chloride, which proceeded in only 15% yield. With very reactive alkylating reagents such as 2,6-dichlorobenzyl bromide, the disodium salt of MTX could be used without addition of caesium carbonate. It may noted that this neutral esterification process also works well with leucovorin and 5-methyltetrahydrohomofolic acid [297], and would undoubtedly have broad applicability for the preparation of lipid soluble diester prodrugs of folate analogues.

Several γ-monoesters of MTX and DCM were also described by Rosowsky *et al.* [237]. Methods of synthesis used to prepare these esters included HCl-catalyzed half-esterification of MTX, partial cleavage of MTX or DCM diesters with base, and mixed anhydride coupling of a γ-monoester of L-glutamic acid with 4-amino-4-deoxy-N^{10}-methylpteroic acid (MeAPA). Half-esterification of MTX afforded mixtures of the diesters and the α- and γ-monoesters, the latter of which were the dominant isomers. Compounds obtained in this initial study were MTX γ-methyl ester (VIII.143), MTX γ-ethyl ester (VIII.144), MTX γ-*n*-butyl ester (VIII.145), and DCM γ-ethyl ester (VIII.146). Pairs of α- and γ-esters could be separated by TLC and column chromatography, the γ-isomers consistently showing higher R_F values consistent with the greater acidity of the α-carboxyl group in comparison with the γ-carboxyl group in α-esters. MTX γ-*t*-butyl ester (VIII.147) was subsequently prepared [299] from MeAPA by condensation with γ-*t*-butyl α-methyl L-glutamate in the presence of diethyl phosphorocyanidate (DEPC) followed by hydrolysis of the methyl ester with $Ba(OH)_2$. In another study [300], the longer-chain γ-*n*-octyl ester (VIII.148), γ-*n*-dodecyl ester (VIII.149), and γ-*n*-hexadecyl ester (VIII.150) were synthesized, along with major amounts of the diesters and smaller amounts of the α-esters, by half-esterification of MTX with the appropriate alkyl bromides in dry DMSO solution in the presence of Cs_2CO_3. Compound (VIII.150) was also synthesized from MTX α-*t*-butyl ester by alkylation with *n*-hexadecyl bromide and Cs_2CO_3 in DMF followed by acidolysis with *p*-toluenesulphonic acid, and from MeAPA by reaction with γ-*n*-hexadecyl α-(2-trimethylsilylethyl) L-glutamate and DEPC followed by deprotection with tetrabutylammonium fluoride.



(VIII.143) $R^1 = R^3 = Me$, $R^2 = H$, X = H
(VIII.144) $R^1 = Me$, $R^2 = H$, $R^3 = Et$, X = H
(VIII.145) $R^1 = Me$, $R^2 = H$, $R^3 = n\text{-}Bu$, X = H
(VIII.146) $R^1 = Me$, $R^2 = H$, $R^3 = Et$, X = Cl
(VIII.147) $R^1 = Me$, $R^2 = H$, $R^3 = t\text{-}Bu$, X = H
(VIII.148) $R^1 = Me$, $R^2 = H$, $R^3 = n\text{-}C_8H_{17}$, X = H
(VIII.149) $R^1 = Me$, $R^2 = H$, $R^3 = n\text{-}C_{12}H_{25}$, X = H
(VIII.150) $R^1 = Me$, $R^2 = H$, $R^3 = n\text{-}C_{16}H_{33}$, X = H
(VIII.151) $R^1 = R^2 = H$, $R^3 = t\text{-}Bu$, X = H
(VIII.152) $R^1 = R^2 = Me$, $R^3 = t\text{-}Bu$, X = H
(VIII.153) $R^1 = CHO$, $R^2 = Me$, $R^3 = t\text{-}Bu$, X = H
(VIII.154) $R^1 = CHO$, $R^2 = H$, $R^3 = t\text{-}Bu$, X = H



(VIII.155) X = OH
(VIII.156) X = Br

In another study aimed at evaluating the activity of esterase stable γ-*t*-butyl esters against MTX-resistant tumour cells, Rosowsky *et al.* [301] resynthesized γ-*t*-butyl MTX (VIII.147) and also prepared γ-*t*-butyl AMT (VIII.151). 2,4-Diamino-6-hydroxymethylpteridine (VIII.155) [302, 303] was brominated with dibromotriphenylphosphorane essentially as described by Piper and Montgomery [247], but isolation and purification of 2,4-diamino-6-bromomethylpteridine hydrobromide ((VIII.156)·HBr) was omitted in favour of condensing the bromide *in situ* with 4-(*N*-methylamino)benzoic acid to obtain 4-amino-4-deoxy-N^{10}-methylpteroic acid (MeAPA) in 90% yield. An improved method of utilization of diethyl phosphorocyanidate was also developed that allowed MeAPA to be condensed with γ-*t*-butyl α-methyl L-glutamate to form the diester, (VIII.154) (75% yield). Hydrolysis of (VIII.152) with $Ba(OH)_2$ in aqueous ethanol then gave monoester (VIII.152) (86% yield). Diester (VIII.152) was also obtained from γ-*t*-butyl α-methyl L-glutamate and (VIII.156)·HBr in the presence of *i*-Pr_2EtN, but the yield was only 10%. When (VIII.156)·HBr was condensed in the same manner with γ-*t*-butyl L-glutamate, the yield of (VIII.147) was 54%. It thus appeared that coupling reactions utilizing the bromide (VIII.156) proceeded more efficiently when the α-COOH group was not esterified, perhaps because this minimized self-condensation.

For the synthesis of the AMT ester (VIII.151), bromide (VIII.156)·HBr was condensed *in situ* with 4-aminobenzoic acid and the product, 4-amino-4-deoxypteroic acid (APA), was treated directly with a mixture of formic and acetic acid to obtain the N^{10}-formyl derivative (FmAPA, 65% yield) [292]. Condensation of FmAPA with γ-*t*-butyl α-methyl L-glutamate to form the diester (VIII.155) was accomplished in 82% yield by a modified mixed anhydride procedure involving four cycles of *in situ* carboxyl group reactivation. Hydrolysis of the methyl ester and N^{10}-formyl group at the same time with NaOH proved impossible as a result of an unforeseen loss of the γ-*t*-butyl ester. When the α-methyl ester was first removed under mild conditions with $Ba(OH)_2$, however, the relatively stable N^{10}-formyl derivative (VIII.154) was isolated satisfactorily in 79% yield. Subsequent cleavage of the N^{10}-formyl group was then accomplished by carefully controlled hydrolysis in 0.25 M NaOH (1.75 h at 25 °C). In an alternative synthesis, FmAPA was activated by reaction with bis(4-nitrophenyl) carbonate to obtain the corresponding 4-nitrophenyl ester (94% yield), which was then converted to (VIII.151) (72%) by reaction with γ-*t*-butyl L-glutamate. This method had the advantage that the need for an ester hydrolysis step was obviated. Ester (VIII.151) was found to be remarkably unstable in comparison with its N^{10}-methyl counterpart, (VIII.147). Thus, it was essential to carefully monitor all bioassay

samples of (VIII.151) for the presence of trace amounts of AMT. After considerable experimentation it was found that, in contrast to the free acid or NH_4 salt, the Na salt of (VIII.151) could be stored safely for several months at $-70\,^{\circ}C$. It was not clear why N^{10}-methyl or N^{10}-formyl substitution would protect the γ-*t*-butyl against hydrolysis, especially at neutral or alkaline pH, where *t*-butyl esters are generally assumed to be stable.

Biological activity

Alkyl diesters of MTX and DCM were reported to have *in vivo* activity against murine L1210 leukaemia by Johns *et al.* [293] and independently by Rosowsky [296] in 1973. Against intraperitoneally (i.p.) implanted tumour, optimal doses of the diethyl (VIII.114), di-*n*-amyl (VIII.119), and di-*n*-octyl (VIII.123) esters given i.p. on a qd × 10 schedule led to increases in lifespan (ILS) of 87, 95 and 128%, respectively, as compared with 99% for MTX [292]. Interestingly, when potencies were compared on a molar basis, the optimal dose of the di-*n*-octyl ester proved to be 50% lower than that of MTX. Activity was also observed against intracerebrally implanted tumour, though ILS values were lower (40–60%). The di-*n*-butyl ester of DCM at 62.5 mg/kg (IP, qd × 4), produced a 66% ILS, an effect comparable to that of DCM on the same schedule [296]. Because these experiments were performed in mice, whose serum esterase activity is high, it was surmised that these straight-chained diesters were being cleaved to MTX monoesters and free MTX. The highly lipophilic diester (VIII.123), in particular, might be expected to have the sustained release properties characteristic of many prodrugs. Support for the idea that the diesters were serving as prodrugs came from the fact that their inhibitory activity against isolated DHFR from L1210 cells was substantially lower than that of MTX. There was, however, an interesting relationship between DHFR inhibition and the length of the alkyl group, the following IC_{50} values (nM) being obtained: (VIII.113) (Me), 380; (VIII.114) (Et), 130; (VIII.117) (*n*-Bu), 5.4; (VIII.119) (*n*-Am), 6.0; (VIII.122) (*n*-Hex), 10; (VIII.123) (*n*-Oct), 130. It was evident that diesters with alkyl groups of medium size (C_4–C_6) were more potent than those with shorter (C_1) or longer (C_8) alkyl groups. The reason for this apparent trend remains unknown. Diesters (VIII.114), (VIII.117), (VIII.122) and (VIII.123) were also tested as thymidylate synthase inhibitors but were devoid of activity [182]. An interesting property of the MTX and DCM diesters was found [293, 295] to be their high substrate activity for hepatic aldehyde oxidase, the enzyme which partially detoxifies MTX and DCM by converting them to 7-hydroxy derivatives [208, 209]. The dimethyl and diethyl esters of MTX had a K_m 50-fold lower that that of MTX itself, and also showed a 2.5-fold higher V_{max}. The dimethyl

ester of DCM similarly had a lower K_m and higher V_{max} than DCM. However, substrate activity was not simply proportional to lipophilicity, since MTX di-*n*-octyl ester had a higher K_m and lower V_{max} than MTX dimethyl ester. While these results did not provide a clear picture of the optimal requirements for aldehyde oxidase substrate activity, they were nonetheless important in showing that this catabolic process could play a major role in the pharmacology of lipophilic derivatives of MTX, and perhaps of other pteridine antifolates as well.

The activity of a large series of MTX and DCM diesters against L1210 leukaemia in mice has been examined by Rosowsky and Yu [297] with the aim of discovering, in particular, whether aralkyl esters might be superior to alkyl esters with regard to the total molar amount of drug required to elicit an antitumour effect. Contrary to what had been found with simple primary alkyl diesters, *ortho*-substituted dibenzyl esters of MTX produced longer survival on a q3d × 3 schedule than did MTX itself, and had a dose-sparing effect comparable to that observed when the parent drug was given at shorter intervals. For example, MTX bis(6-chloropiperonyl) ester (VIII.142) at a dose equivalent to 5.5 mg/kg of MTX gave an ILS of 88%, whereas for MTX itself 30 mg/kg was required to achieve the same ILS on the q3d × 3 schedule. When the diester was given at a dose equivalent to 40 mg/kg of MTX a 167% ILS was obtained. By comparison, 60 mg/kg of MTX gave only a 100% ILS. High activity (> 100% ILS) was also observed for the MTX bis(aralkyl) esters (VIII.137), (VIII.139) and (VIII.140), and for the DCM bis(2-amyl) ester (VIII.129). It thus appeared that, contrary to earlier indications, diesters of MTX (and of DCM) were *not all therapeutically equivalent* in mice, despite the high serum esterase activity in this species, and that a reduction in total administered molar dose of as much as 10-fold could be achieved on the q3d × 3 schedule by substituting a diester for the parent acid. It should be noted, however, that while these results were consistent with the idea that the diesters were acting simply as slow release forms of the parent acid, the possibility could not be excluded that some of the antitumour activity of the diesters was due to the formation of γ-monoesters. As discussed below, γ-monoesters of MTX bind almost as tightly as MTX to DHFR and are active against tumour cells in culture. It was also possible that the dose-sparing effect observed with the diesters reflected the formation of sparingly soluble depots in the peritoneal cavity, which might be expected to simulate, and be pharmacokinetically equivalent to, slow intravenous infusion.

The high lipophilicity of the MTX diesters made these compounds attractive candidates for use against MTX resistant tumours whose phenotypes include a transport defect. In the first test of the potential validity of this

approach in overcoming MTX resistance [304], MTX and MTX di-*n*-butyl ester (DBMTX, VIII.117) were compared as inhibitors of the growth of CEM cells and of a resistant subline (CEM/MTX) whose DHFR content was normal, but whose ability to take up MTX was markedly impaired. While the IC_{50} values of MTX against the parental CEM cells were 0.029 and 3.4 μM, respectively, the corresponding values for DBMTX were found to be 0.069 and 0.012 μM. Thus, the CEM/MTX cells were 120-fold resistant to MTX but 'collaterally sensitive' to DBMTX, just as they were to more conventional lipid-soluble antifolates like 2,4-diamino-5-(3,4-dichlorophenyl)-6-methylpyrimidine (DDMP, metoprine). The diester was, in fact, more potent against the resistant cells than MTX itself was against the parental line. The basis for this collateral sensitivity was suggested to be the fact that the CEM/MTX were probably less efficient than the parental line in taking up reduced folates from the growth medium, which increased their sensitivity to lipophilic antifolates that bypass the transport pathway shared by classical antifolates and natural reduced folates. A second possible mechanism for DBMTX was also suggested by the earlier finding that this compound interfered with the uptake of both [^{3}H]dUrd and [^{3}H]dThd into DNA, whereas MTX inhibited [^{3}H]dUrd incorporation but, as expected, had no effect on [^{3}H]dThd incorporation [305]. That this was mediated at the level of dThd uptake was subsequently demonstrated by the finding of a substantial decrease in the total acid soluble pool of dTMP, dTDP, and dTTP when L1210 cells were incubated with [^{3}H]dThd and DBMTX, but not MTX [306]. Since thymidine kinase activity in a cell-free assay was not affected by DBMTX, it was concluded that the effect was not due to inhibition of dThd phosphorylation. A decrease in incorporation of [^{3}H]uridine and [^{3}H]deoxyuridine into the acid-soluble pool of the uridine and deoxyuridine nucleotides was similarly noted. It thus appeared that, in addition to being able to enter cells efficiently by a pathway alternative to the one used by MTX, the diester also had the potential to interfere with dThd uptake, and hence salvage. The mechanism by which nucleoside uptake is inhibited by DBMTX is unknown. Indeed, the mechanism of uptake of the MTX diesters themselves has not been elucidated, and is likely to be difficult to study kinetically because of the high tendency of these compounds to bind to the cell membrane, as is true also for conventional lipid soluble antifolates like DDMP [307]. It is of interest that tumour cells pleiotropically resistant to anthracyclines and vinca alkaloids have been found to be cross-resistant to MTX diesters, including DBMTX [308]. This suggests that DBMTX may use the same transport pathway as these lipophilic natural products.

The ability of DBMTX (VIII.117) to overcome transport-based resistance

has also been documented recently [258]. Whereas the IC_{50} value of this compound against a transport and polyglutamylation defective human head and neck squamous cell carcinoma line (SCC15/R1) was comparable to its IC_{50} value against the MTX-sensitive parental line (SCC15), the difference in IC_{50} values for MTX was 17-fold. It was also noted in this study that, in SCC sublines whose level of MTX resistance was increased further (up to 9000-fold) by virtue of a combination of impaired transport and increased DHFR content, resistance to DBMTX was consistently lower than resistance to MTX. For example, the subline SCC15/R3, whose rate of MTX uptake was only one-sixth of that of SCC15 cells and whose *dhfr* gene was amplified 4.4-fold, displayed 730-fold resistance to MTX but only 9-fold resistance to DBMTX. It thus appeared that the ease with which the lipophilic diester was taken up could compensate for the fact that more target enzyme was present in the cell. Studies on the ability of MTX diesters other than DBMTX to overcome MTX resistance in cultured cells have not been reported, and might be of interest.

A potentially important use of MTX diesters has been proposed to be in the treatment of tumours of the central nervous system [292]. Rosowsky *et al.* [309] examined the pharmacokinetics and metabolism of DBMTX in Rhesus monkeys, and demonstrated that when only free (*i.e.*, not protein-bound) drug was considered, the CSF/plasma ratio for the diester, as well as for its major metabolite, MTX γ-*n*-butyl ester, was indeed higher than the ratio for MTX. However, when account was taken of the fact that binding to plasma proteins was 90–95% for DBMTX as compared to only 50% for MTX, the ratios of total drug in the CSF and plasma compartments for the two compounds were not very different. A greater fraction of the injected dose of ester was excreted in bile than in urine, whereas the opposite was true for MTX. This was consistent with the idea that hepatic extraction is favoured for the lipophilic diester derivative in comparison with the more water-soluble parent acid.

Another potential use of MTX diesters suggested [310, 311] on the basis of the high lipophilicity of these compounds has been in the percutaneous treatment of psoriasis [256]. Although several MTX diesters were tested by skin application in various vehicles, the results were mostly discouraging.

Rosowsky *et al.* [237] reported in 1978 that the MTX γ-monoesters (VIII.144) (Et) and (VIII.145) (*n*-Bu) inhibited the growth of human leukaemic lymphoblasts (CEM cells) in culture (IC_{50} = 0.58 and 0.76 μM) but were less potent on a molar basis than the corresponding diesters (IC_{50} = 0.012–0.057 μM) and even less potent than MTX itself (IC_{50} = 0.006 μM). When (VIII.144) was compared with DBMTX and MTX as an inhibitor of isolated DHFR, on the other hand, the monoester

(IC_{50} = 17 nM) was found to be more potent than the diester (IC_{50} = 140 nM) and only 2-fold less potent than the diacid (IC_{50} = 9 nM). From this it was concluded that MTX γ-*n*-butyl MTX had the potential to inhibit the growth of tumour cells in culture as effectively as MTX, but presumably was not taken up as well as either MTX or DBMTX. It was also demonstrated in this study that the stability of DBMTX toward serum esterase cleavage to MTX was species-variable. Thus, incubation of (VIII.144) with mouse serum for 48 h resulted in 93% cleavage to MTX, whereas a similar incubation in monkey serum produced only 2% MTX, 27% of the γ- and α-monoesters (85:15 mixture according to HPLC analysis), and 71% of unchanged diester. In human serum, cleavage was even less extensive, with 88% unchanged diester, 12% monoester mixture, and < 1% MTX. These results showed for the first time that monoesters of MTX could be expected to play a role in the pharmacology of diesters in non-rodent species.

A possibility that always has to be considered with antifolates whose DHFR affinity is relatively low is whether inhibition of cell growth is due to interference with the activity of another enzyme such as thymidylate synthase. In assays measuring the ability of MTX γ-*n*-butyl ester (VIII.144) to inhibit purified thymidylate synthase (TS) from human cells [182], an estimated K_i of 40 μM was obtained, as compared with 30 μM for MTX. It was therefore very unlikely that TS inhibition contributed to the activity of the ester against cultured cells. The fact that γ-substitution did not have much effect on TS binding was of interest, in that it suggested a similarity between DHFR and TS with respect to the 'terminal region' of the folate ligand. In contrast to the monoester (VIII.144), DBMTX was virtually devoid of activity, demonstrating the importance of a free α-COOH group for binding to this enzyme.

In a more recent study on MTX γ-monoesters [239], (VIII.144) was compared with the longer-chain monoesters (VIII.148) (*n*-C_8H_{17}), (VIII.149) (*n*-$C_{12}H_{25}$), and (VIII.150) (*n*-$C_{16}H_{33}$) with respect to DHFR inhibition and cell growth inhibition. While (VIII.144) (IC_{50} = 5.6 nM) and (VIII.148) (IC_{50} = 5.4 nM) were almost as potent as MTX (IC_{50} = 3.3 nM) as inhibitors of crude bovine liver enzyme, the longer-chain analogues (VIII.149) (IC_{50} = 34 nM) and (VIII.150) (IC_{50} = 37 nM) were approximately 10-fold less potent. This suggested that while small γ-ester groups are well tolerated, there may exist on the active-site surface a domain not compatible with *long* hydrophobic substituents at the γ-position of the glutamate moiety. In general the γ-esters were 10-fold more potent than the α-esters (*vide supra*), confirming the importance of a free α-COOH group for binding. A notable feature of the cell culture data (CEM hyman lymphoblasts) was that the monoesters (VIII.149) (IC_{50} 0.37 μM) and (VIII.150) (IC_{50} = 0.11 μM) were more potent

than the shorter-chained esters (VIII.144) (IC_{50} = 0.78 μM) and (VIII.148) (IC_{50} = 0.92 μM), despite the fact that the latter were the better DHFR inhibitors. This suggested that esters with more lipophilic long-chain alkyl groups might be accumulating more efficiently in cells. With more free drug in excess of the amount needed to just saturate the target enzyme, dissociation of the enzyme-inhibitor complex would be minimized. It was also speculated that long-chain alkyl substituents might promote binding to lipid-rich sites in the cell, thereby retarding efflux and, in a sense, simulating polyglutamylation.

Of all the monoesters, probably the most interesting have been MTX γ-*t*-butyl ester (VIII.147) and AMT γ-*t*-butyl ester (VIII.151). Both compounds are good inhibitors of purified DHFR from L1210 cells, with IC_{50} values of 55 and 22 nM, respectively, versus 67 and 25 nM for MTX and AMT [301]. In growth inhibition assays against human leukaemic lymphoblasts (CEM cells), (VIII.147) had an IC_{50} of 0.62 μM (versus 0.032 μM for MTX), while (VIII.151) had an IC_{50} value of 0.45 μM (versus 0.001 μM for AMT). Thus the AMT ester was more potent than the MTX ester, just as AMT was more potent than MTX. Against the transport-defectivethe IC_{50} for (VIII.147) was 0.5 μM (versus 6.6 μM for MTX), while the IC_{50} value for (VIII.151), unexpectedly, was 25 μM (versus 0.32 μM for AMT). Thus, the ester of MTX was almost equally inhibitory to the MTX-resistant and MTX-sensitive cells, whereas the ester of AMT was much less active against MTX-resistant cells than against MTX-sensitive cells. Thus γ-esterification influenced biological activity quite differently depending on the nature of the N^{10} substituent. The reason for this was unclear, though similar inconsistencies have been observed in several other pairs of MTX and AMT analogues modified in the γ-terminal region, as discussed elsewhere in this review. When (VIII.147) and (VIII.151) were compared as inhibitors of L1210 cell growth, IC_{50} values of 0.0056 and 0.023 μM, respectively were obtained, as compared with 0.002 μM for both MTX and AMT. As expected, the highly MTX-resistant L1210/R81 cell line, which is profoundly defective in MTX transport [48], was only partly cross-resistant to the esters. Interestingly, another MTX-resistant subline, L1210/R71, whose resistance is associated with increased DHFR activity, likewise showed only partial cross-resistance to the esters. Incomplete cross-resistance was similarly observed in three MTX resistant lines of human head and neck squamous cell carcinoma. The AMT ester (VIII.151) was consistently more potent than the MTX ester (VIII.151) against MTX-sensitive as well as MTX-resistant cells. For example, the IC_{50} values of (VIII.147) against the MTX-sensitive line SCC25 and the MTX-resistant subline SCC25/R1 [258] were 0.40 and 0.78 μM, respectively, whereas the corresponding values for (VIII.151) were 0.066 and 1.8 μM. The SCC25/R1 cells

were 11-fold resistant to MTX, but only 2-fold cross-resistant to the MTX ester, showing again that esterification was able to at least partially overcome resistance in these cells. It appeared that incomplete cross-resistance to the *t*-butyl ester of MTX occurred irrespective of whether resistance to the parent acid was due primarily to a transport defect or to increased DHFR content.

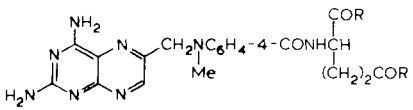
In assays of *in vivo* antitumour activity against L1210 leukaemia in mice, MTX γ-*t*-butyl ester (VIII.147) was found to produce an 87% ILS at its optimal dose of 60 mg/kg (bid × 10), while AMT γ-*t*-butyl ester (VIII.151) gave a 73% ILS at its optimal dose of 12 mg/kg [301]. The ester of AMT was therefore more potent than the ester of MTX, as is the case for the parent acids. However, both esters were substantially less potent than the respective acids, since the doses of MTX and AMT sufficing to give a 70–80% ILS on the bid × 10 schedule had been found earlier to be 0.5 and 0.12 mg/kg. Several possible reasons could, in principle, account for the profound discrepancy between the *in vitro* and *in vivo* activity of the esters and parent acids. One was that the pharmacokinetics of the esters were unfavourable, that is, that the area under the curve of plasma concentration versus time was lower for the esters than for the parent acids. That this was likely, at least for (VIII.147), was suggested by the fact that the plasma half-life for this compound after a bolus i.p. injection was lower than that of MTX given at the same dose [299]. While increased plasma clearance could be due to more rapid renal excretion, it was also suspected that the lipophilicity of the ester might favour extraction by the liver and subsequent metabolism to a 7-hydroxy derivative by hepatic aldehyde oxidase. Support for this idea came from measurement of the substrate activity of (VIII.147) toward partially purified aldehyde oxidase from rabbit liver [263]. In a side-by-side comparison of the kinetics of 7-hydroxylation of MTX and (VIII.147), the K_m values were found to be 189 ± 58 and 24 ± 1.4 μM, respectively, while the V_{max} values were 30 ± 9 and 240 ± 11 nmol/mg protein per min. The efficiency of 7-hydroxylation of the ester ($V_{max}/K_m = 10$) was therefore 60-fold higher than that of MTX ($V_{max}/K_m = 0.16$). From this it was concluded that the major reason for the discrepancy between the *in vitro* and *in vivo* activity of (VIII.147) was that this compound was extensively detoxified in the liver. This has been demonstrated to also be the case for 3′,5′-dichloromethotrexate (DCM), whose molar potency *in vivo* is substantially lower than that of MTX because of more rapid 7-hydroxylation [208, 209]. 7-Hydroxylation of γ-*t*-butyl DCM has also been observed, and has been found to be more rapid than the reaction of γ-*t*-butyl MTX [312], as has been confirmed recently [313] for DCM ($K_m = 9.6\ \mu M$, $V_{max}/K_m = 24$) versus MTX ($K_m = 34.5\ \mu M$, $V_{max}/K_m = 7.2$) and AMT ($K_m = 72\ \mu M$, $V_{max}/K_m = 0.48$). It would appear that, in the design

of MTX analogues with lipophilic side-chains, consideration should always be given to the possibility of accelerated detoxification by hepatic aldehyde oxidase. An obvious way to circumvent this problem would, of course, be to replace N^8 by a carbon atom as in compounds of the 8-deaza or 5,8-dideaza family. One might imagine, on the other hand, that efficient 7-hydroxylation would be advantageous under some circumstances, for example, in the treatment of liver metastatic disease by intrahepatic arterial infusion, where local detoxification would have a sparing effect on distant host tissues. Lipophilic γ-esters of DCM would be logical candidates to consider using in this context.

γ-Amides and α,γ-diamides

Chemical synthesis

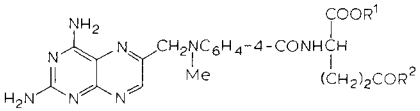
A series of lipophilic diamides ((VIII.157)–(VIII.169)) were prepared from MTX diesters [314] by heating the diesters for 24–72 h at 50–100 °C in the presence of excess amine, with or without solvent. Yields with primary amines were generally in excess of 50% except for the reactions with ammonia itself and with 2-*N*,*N*-dimethylaminoethylamine, which gave somewhat lower yields. Yields were also lower with secondary amines. Interestingly, when MTX itself, as the Na salt, was heated with *n*-propylamine in ethanolic HCl, a 78% yield of diamide was obtained in one operation, probably via transient esterification. Under forcing conditions, replacement of the amino groups on the pteridine ring was also noted. Thus, heating MTX dimethyl ester with benzylamine at 185 °C for 38 h afforded the 2,4-di(*N*-benzyl) derivative of MTX dibenzylamide (15% yield). When the reaction was attempted with MTX diethyl ester and piperidine in 95% ethanol, only MTX γ-ethyl ester was



(VIII.157)	R = NH_2	(VIII.168)	R = $NHCH_2CH_2CH_2OH$
(VIII.158)	R = $NHCH_2CH_2NMe_2$	(VIII.169)	R = $NHCH_2CH_2CH_2NMe$
(VIII.159)	R = NH(*n*-Pr)	(VIII.170)	R = $NHNH_2$
(VIII.160)	R = NH(*n*-Bu)	(VIII.171)	R = $NMeNH_2$
(VIII.161)	R = NH(s-Bu)	(VIII.172)	R = NH(1-adamantyl)
(VIII.162)	R = NH(*n*-Hex)	(VIII.173)	R = piperidino
(VIII.163)	R = NH(c-C_6H_{11})	(VIII.174)	R = $NHCH_2C_6H_4$(4-Cl)
(VIII.164)	R = pyrrolidino	(VIII.175)	R = $NHCH_2C_6H_4$(4-OMe)
(VIII.165)	R = $NHCH_2Ph$	(VIII.176)	R = $NMeCH_2Ph$
(VIII.166)	R = $NHCH_2CH_2Ph$	(VIII.177)	R = $N(CH_2Ph)_2$
(VIII.167)	R = $NHCH_2CH_2C_6H_3$-(3,4-OMe)$_2$	(VIII.178)	R = NHCHMePh
		(VIII.179)	R = NHPh

recovered. In a subsequent study [315] the dihydrazide derivatives (VIII.170) and (VIII.171) were also prepared from MTX diethyl ester. The structure of (VIII.171) was not unequivocally proved, but was consistent with other acylation reactions of *N*-methylhydrazine, which occur preferentially on the more basic alkyl nitrogen. Eight other diamides (VIII.172)–(VIII.179) were also prepared from 4-amino-4-deoxy-N^{10}-methylpteroic acid and appropriate L-glutamic acid diamides via carbodiimide or mixed anhydride condensation reactions.

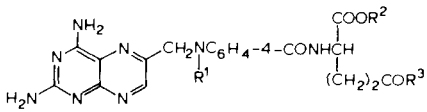
γ-Monoamides of MTX have been described independently by Rosowsky *et al.* [299], Piper *et al.* [240], and Antonjuk *et al.* [241, 242]. Condensation of 4-amino-4-deoxy-N^{10}-methylpteroic acid (MeAPA) with α-benzyl L-glutamic acid γ-*n*-butylamide and α-benzyl L-glutamic acid γ-benzylamide in the presence of diethyl phosphorocyanidate afforded 70–80% yields of the ester amides (VIII.180) and (VIII.181), which on hydrolysis with $Ba(OH)_2$ in aqueous ethanol afforded MTX γ-*n*-butylamide (VIII.182) (73%) and MTX γ-benzylamide (VIII.183) (88%), respectively [299]. Other MTX γ-amides have been prepared from α-*t*-butyl *N*-[4-(*N*-methylamino)benzoyl]-L-glutamic acid γ-amides by reaction with 2,4-diamino-6-bromomethylpteridine hydrobromide ((VIII.156)·HBr) followed by acidolysis in trifluoroacetic acid (route A) [240, 241], and in one instance by reaction with 2-amino-5-bromomethyl-3-cyanopyrazine 1-oxide followed by deoxygenation with $(EtO)_3P$ in hot DMF, annulation with guanidine, and acidolysis (route B) [241]. Compounds obtained via route A included the γ-*n*-propylamide (VIII.184), γ-piperidide (VIII.185), (VIII.185), γ-cyclohexylamide (VIII.186), and γ-mor-



(VIII.180) R^1 = $PhCH_2$, R^2 = NH(*n*-Bu)
(VIII.181) R^1 = R^2 = CH_2Ph
(VIII.182) R^1 = H, R^2 = NH(*n*-Bu)
(VIII.183) R^1 = H, R^2 = $NHCH_2Ph$
(VIII.184) R^1 = H, R^2 = NH(*n*-Pr)
(VIII.185) R^1 = H, R^2 = piperidino
(VIII.186) R^1 = H, R^2 = cyclohexylamino
(VIII.187) R^1 = H, R^2 = morpholino
(VIII.188) R^1 = H, R^2 = NH_2
(VIII.189) R^1 = *t*-Bu, R^2 = NH(*n*-Pr)
(VIII.190) R^1 = *t*-Bu, R^2 = piperidino
(VIII.191) R^1 = *t*-Bu, R^2 = cyclohexylamino
(VIII.192) R^1 = *t*-Bu, R^2 = morpholino
(VIII.193) R^1 = H, R^2 = NHMe
(VIII.194) R^1 = H, R^2 = NMe_2

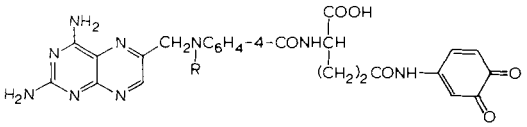
pholide (VIII.187), along with the intermediate α-*t*-butyl esters (VIII.189)–(VIII.192). Compounds prepared via route B were the α-*t*-butyl ester (VIII.192) and the deprotected γ-amide (VIII.188), the latter of which had also been made earlier from (VIII.156)·HBr [240]. Compounds (VIII.180), (VIII.181), and the *N*-methyl and *N,N*-dimethyl analogues (VIII.192) and (VIII.194) were also prepared [240] from (VIII.156)·HBr and the appropriate *N*-[4-(*N*-methylamino)benzoyl]-L-glutamic acid γ-amides without α-carboxyl protection. The major difference between protection of the α-carboxyl and lack of such protection lies, of course, in the fact that unprotected γ-amides can be purified on an ion-exchange column, whereas the amide esters require silica-gel chromatography.

In more recent work involving a series of alkyl, aralkyl and arylamides of MTX and AMT [316], MeAPA was condensed with α-benzyl L-glutamic acid γ-3,4-methylenedioxyanilide by the diethyl phosphorocyanidate coupling procedure to obtain the ester amide (VIII.195). The benzyl ester group in (VIII.195) was then removed with NaOH to form (VIII.196) in 64% overall yield. Further reaction of (VIII.196) with boron tris(trifluoroacetate) in trifluoroacetic acid at 0 °C for 15 min afforded MTX γ-(3,4-dihydroxyanilide) ((VIII.197) 79% yield). AMT γ-amides (VIII.198)–(VIII.205) were prepared via the blocked intermediates (VIII.206)–(VIII.213), which were generated from FmAPA by the modified mixed anhydride route (four activation cycles;



(VIII.195) R^1 = Me, R^2 = $PhCH_2$, R^3 = $NHC_6H_3(3,4\text{-}OCH_2O)$
(VIII.196) R^1 = Me, R^2 = H, R^3 = $NHC_6H_3(3,4\text{-}OCH_2O)$
(VIII.197) R^1 = Me, R^2 = H, R^3 = $NHC_6H_3(3,4\text{-}OH)_2$
(VIII.198) R^1 = R^2 = H, R^3 = NH(*t*-Bu)
(VIII.199) R^1 = R^2 = H, R^3 = NH(1-adamantyl)
(VIII.200) R^1 = R^2 = H, R^3 = $NHCH_2Ph$
(VIII.201) R^1 = R^2 = H, R^3 = $NHCH_2C_6H_4(4\text{-}Cl)$
(VIII.202) R^1 = R^2 = H, R^3 = $NHCH_2C_6H_3(3,4\text{-}Cl_2)$
(VIII.203) R^1 = R^2 = H, R^3 = $NHCH_2C_6H_3(2\,6\text{-}Cl_2)$
(VIII.204) R^1 = R^2 = H, R^3 = NHC_6H_5
(VIII.205) R^1 = R^2 = H, R^3 = $NHC_6H_3(3,4\text{-}OCH_2O)$
(VIII.206) R^1 = CHO, R^2 = $PhCH_2$, R^3 = NHBu-*t*
(VIII.207) R^1 = CHO, R^2 = $PhCH_2$, R^3 = NH(1-adamantyl)
(VIII.208) R^1 = CHO, R^2 = $PhCH_2$, R^3 = $NHCH_2Ph$
(VIII.209) R^1 = CHO, R^2 = $PhCH_2$, R^3 = $NHCH_2C_6H_4(4\text{-}Cl)$
(VIII.210) R^1 = CHO, R^2 = $PhCH_2$, R^3 = $NHCH_2C_6H_3(3,4\text{-}Cl_2)$
(VIII.211) R^1 = CHO, R^2 = $PhCH_2$, R^3 = $NHCH_2C_6H_3(2,6\text{-}Cl_2)$
(VIII.212) R^1 = CHO, R^2 = $PhCH_2$, R^3 = NHC_6H_5
(VIII.213) R^1 = CHO, R^2 = $PhCH_2$, R^3 = $NHC_6H_3(3,4\text{-}OCH_2O)$
(VIII.214) R^1 = R^2 = H, R^3 = $NH_6H_3(3,4\text{-}OH)_2$

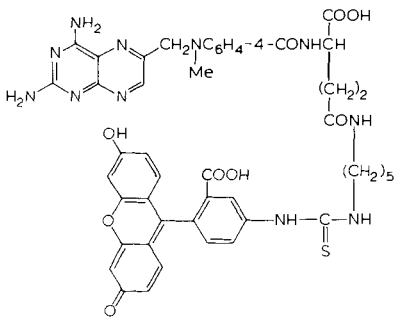
vide supra). Cleavage of the methylenedioxy group in (VIII.205) boron bis(trifluoroacetate) afforded AMT γ-(3,4-dihydroxyanilide) ((VIII.214), 85% yield). The γ-(2,6-dichlorobenzylamide) (VIII.211) was also obtained from FmAPA by the nitrophenyl ester activation method.



(VIII.215) R = Me
(VIII.216) R = H

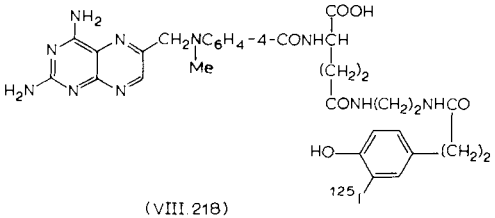
The γ-(3,4-dihydroxyanilides) (VIII.197) and (VIII.214) were made [316] on the novel basis that they might undergo intracellular oxidation to *ortho*-quinones (VIII.215) and (VIII.216), respectively. The latter would have the potential, in principle, to react covalently with nucleophilic groups in the DHFR active site by Michael addition.

Several derivatives of MTX containing some type of bifunctional γ-amide 'spacer' have been described. One of these is the fluorescein derivative (VIII.217) ('MTX-F'), which was obtained, along with the corresponding α-isomer, by allowing MTX to react with 4′-(5-aminopentanylthiocarbamoyl)fluorescein in dry DMSO containing 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide (EDC) hydrochloride [317–320]. The desired γ-isomer was isolated in about 40% yield after purification on DEAE-cellulose. The use of this compound as a fluorescent DHFR probe has been reported in a number of papers [319–325]. Its characteristics are generally similar to those of another fluorescent MTX analogue called PT430, which was synthesized independently by Rosowsky *et al.* [326] and is described in the next section.

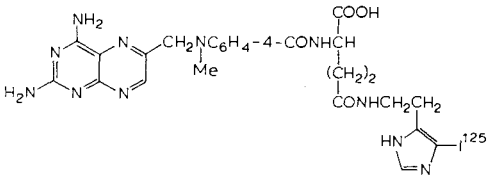


(VIII.217)

Condensation of MTX with ethylenediamine in DMF solution in the presence of EDC hydrochloride was reported by Kamel and Gardner [327] to give an amide derivative, which presumably consisted of a mixture of α- and γ-isomers. Purification by preparative paper chromatography afforded a compound which on coupling to ^{125}I-labeled *N*-succinimidyl-3-(4-hydroxyphenyl)propionate (Bolton-Hunter reagent) afforded a product suitable for use in MTX radioimmunoassays based on competitive binding to anti-MTX immunoglobulin. Although formal proof that this product was γ-substituted was not offered, structure (VIII.218) may be provisionally assigned. Kamel and Gardner [327] also condensed MTX with radioiodinated histamine by the mixed anhydride method (*i*-BuOCOCl-*n*-Bu_3N) to obtain, after chromatography on Sephadex LH-20 a product which most probably consisted of a mixture of the γ-amide (VIII.219) and its α-isomer. This preparation was similarly used in the development of a sensitive radioimmunoassay for MTX.

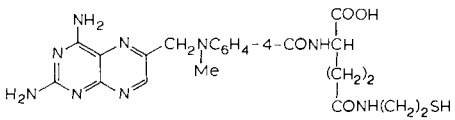


(VIII.218)



(VIII.219)

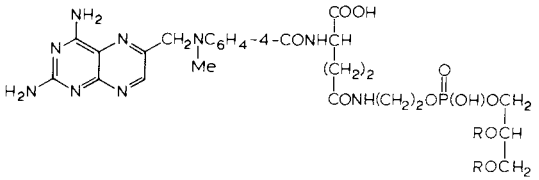
A putative γ-amide derivative was also prepared from MTX by reaction with cystamine in water solution in the presence of EDC hydrochloride, followed by reduction of the disulphide bond with excess 2-mercaptoethanol [328]. The resulting thiol (VIII.220) was then allowed to react with



(VIII.220) (isolated as the disulphide)

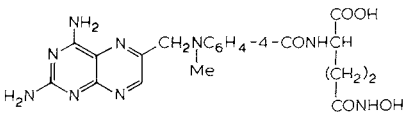
poly(D-lysine) modified by 3-(2-pyridyldithio)propionylation in order to obtain a novel MTX-'spacer'-poly(D-lysine) conjugate.

A more complex type of γ-amide derivative has been prepared from MTX by reaction with dimyristoylphosphatidylethanolamine in the presence of *N*-hydroxysuccinimide and dicyclohexylcarbodiimide [329]. The main product (17.5% yield) was identified as the γ-substituted derivative (VIII.221). Smaller amounts of the α, γ-diamide and α-amide were also obtained. Structure assignments were made on the basis of thin-layer chromatographic mobility, with the diamide being more mobile than either of the monoamides and the α-amide being more mobile than the γ-amide. Gentle alkaline hydrolysis of (VIII.221) afforded the glycerophosphatidylethanolamide (VIII.222) in 69% yield. Once again, this compound was less mobile on silica gel than its α-isomer. It appears to be a general property of MTX analogues with a free α-COOH group that they are more polar than those containing a free γ-COOH group [239, 330]. Compound (VIII.221) was used to prepare liposomes in which MTX γ-dimyristoylglycerophosphatidylethanolamide moieties were embedded in the lipid bilayer.



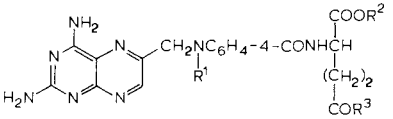
(VIII.221) R = $CH_3(CH_2)_{12}CO$
(VIII.222) R = H

An analogue of MTX in which the γ-COOH group was replaced by γ-CONHOH was described in 1981 [314]. Treatment of MTX diethyl ester with hydroxylamine in the presence of NaOMe afforded the γ-hydroxamic acid derivative (VIII.223) (81% yield). As expected from its structure, (VIII.223) gave a reddish violet colour on reaction with $FeCl_3$. The location of the NHOH group at the γ-position was confirmed by showing that the same product was formed, albeit in low yield (8%), from MeAPA and L-glutamic acid γ-hydroxamate (non-protected) on mixed anhydride coupling.



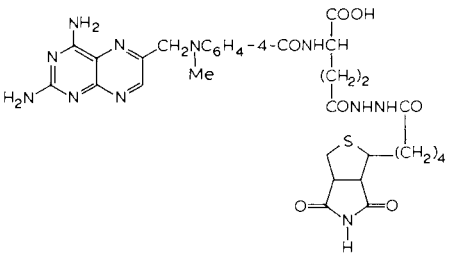
(VIII.223)

A synthesis of MTX γ-hydrazide (VIII.224) was described in 1981 by Rosowsky *et al.* [299]. Condensation of MeAPA with γ-methyl α-*t*-butyl L-glutamate with the aid of diethyl phosphorocyanidate afforded the diester (VIII.225) (76% yield), which was then converted sequentially to (VIII.226) (60% yield) on treatment with hydrazine in MeOH solution at 4 °C for 3 days and to (VIII.224) (45% yield) on hydrolysis with 1 M HCl at 50 °C for 1 h. Compound (VIII.224) has gained attention recently because of the opportunity it affords to couple MTX to monoclonal antitumour antibodies through the formation of semicarbazide bonds to periodate-oxidized sugars [331]. The γ-hydrazide derivative (VIII.227) of AMT has also been cited briefly [332], although details of its synthesis have not yet been disclosed.



(VIII.224) R^1 = Me, R^2 = H, R^3 = $NHNH_2$
(VIII.225) R^1 = R^3 = Me, R^2 = *t*-Bu
(VIII.226) R^1 = Me, R^2 = *t*-Bu, R^3 = $NHNH_2$
(VIII.227) R^1 = H, R^2 = $NHNH_2$

Another γ-hydrazide derivative of MTX was prepared by Rosowsky and Forsch [333] from α-*t*-butyl MTX by a mixed anhydride activation of the γ-carboxyl group (*i*-BuOCOCl-Et_3N) followed by addition of biotin hydrazide and treatment with trifluoroacetic acid (room temperature, 10 min) to obtain the biotinyl derivative (VIII.228). The overall yield for the coupling and deprotection steps was approximately 30%.



(VIII.228)

Biological activity

In vitro assays on MTX diamides have been limited to the testing of a few selected examples against cultured tumour cells [314] and as inhibitors of DHFR [237] and TS [182]. Diamides (VIII.158) (n-C_3H_7), (VIII.161) (n-C_6H_{13}), (VIII.162) (c-C_6H_{13}), and (VIII.164) (CH_2Ph) were found to inhibit the growth of human leukaemic lymphoblasts (CEM cells) with IC_{50} values of 1–10 μM as compared with 0.003 μM for MTX [314]. Rat basophilic leukaemia cells, which are myeloid rather than lymphoid in origin, were also tested on the basis that they might possess higher amidase activity [334]. As predicted, a 3- to 5-fold increase in potency against these cells was observed. However, it was evident that, as a class, diamides of MTX were far less active than diesters in an *in vitro* setting. In this regard it is worth noting that α-amide substitution is more detrimental to enzyme binding than γ-amide substitution. For example, it has been reported [242] that the IC_{50} values of MTX, MTX γ-amide, MTX α-amide, and MTX α,γ-diamide as inhibitors of *L. casei* DHFR are 2.7, 24 and 260 nM, respectively. From calculated K_i values and the equation $\Delta G_{app} = -RT\ln(1/K_i)$ for the binding energy, it was estimated that γ-amidation resulted in a loss of binding energy of only 5.4 kJ/mol relative to MTX, whereas for α-amidation this loss was 11.4 kJ/mol. Diamidation therefore produces very little additional loss of binding energy. While the corresponding data are not available for mammalian DHFR, it is likely that this qualitative trend also obtains for the latter enzyme. In the case of human TS [182], MTX α-amide and the substituted α,γ-diamides (VIII.159) and (VIII.162)–(VIII.164) were shown to be very poor inhibitors ($K_i > 300\ \mu$M), whereas several substituted γ-monoamides (*vide infra*) had estimated K_i values in the 30–40 μM range and were thus equipotent with MTX. Although these K_i values are still too high to make TS inhibition relevant to the biological action of these compounds, they do illustrate the point that γ-substitution is less detrimental than α-substitution with regard to the binding of inhibitors to both enzymes.

While the *in vitro* data on MTX diamides were generally discouraging, one compound, the dibenzylamide (VIII.164), nonetheless showed moderate activity against L1210 leukaemia in mice (40–80% ILS at 100 mg/kg, q3d × 3) [314]. A notable feature of these results was that, in sharp contrast to MTX diesters, no MTX was formed on *in vitro* incubation of (VIII.164) with mouse serum. However, HPLC analysis of the plasma of mice treated with the dibenzylamide revealed that free MTX was formed in substantial amounts *in vivo*. Thus, this was the first example of a MTX prodrug whose bioactivation occurred elsewhere than in plasma. That the nature of the amide substituent plays a critical role in this bioactivation was demonstrated subsequently by

Rosowsky *et al.* [314], who reported significant *in vivo* activity for MTX α,γ-dihydrazide (VIII.170), MTX α,γ-di(4-methoxybenzylamide) (VIII.175), and MTX α,γ-dianilide (VIII.179). Treatment of L1210 leukaemic mice with 120 mg/kg of the dianilide (VIII.179) (equivalent to 90 mg/kg of MTX) on the q3d × 3 schedule produced a 155% ILS, as compared with ILS values of 83 and 100% for MTX at 30 and 60 mg/kg. Treatment with 20 mg/kg of the dihydrazide (VIII.170) produced a 77% ILS. It was concluded that certain of the MTX diamides were especially prone to enzymatic bioactivation, and that this could be a potentially fruitful area of further exploration.

Bioassay data for the MTX γ-monoamides (VIII.182) (*n*-BuNH) and (VIII.183) ($PhCH_2NH$) were reported independently by Rosowsky *et al.* [299] and by Piper *et al.* [240]. Data for the amides VIII.188 (NH_2), VIII.193 (MeNH), and VIII.194 (Me_2N) were also reported [240]. All five compounds were potent inhibitors of DHFR from L1210 cells, with estimated K_i values of 2.7–3.9 pM as compared with 4.3 pM for MTX. Thus, conversion of the γ-carboxyl group to an amide clearly had very little effect on binding to DHFR, and, if anything, seemed to promote it. The potency of the γ-amides (VIII.182) and (VIII.183) against DHFR from *L. casei*, on the other hand, was *ca.* 3-fold lower than that of MTX [299], illustrating once again that the effect of structural modifications of MTX always has to be evaluated against a relevant enzyme.

Uptake of the amides into L1210 cells, was considerably slower than that of the parent acid, as indicated by K_m values for unidirectional influx (measured by titration of drug in cell lysates with DHFR) or K_i values for competitive inhibition of [^{3}H]MTX transport [240]. The K_m values of the amides ranged from 3.3 μM for (VIII.188) to 48 μM for (VIII.194), while the K_m for MTX uptake by these cells was 3.3 μM. First-order rate constants for efflux, on the other hand, were hardly affected by γ-substitution. Transport into the cells was therefore predicted to be the limiting event for cell growth inhibition, and this was experimentally borne out by growth inhibition assays with H.Ep.2 cells, against which the amides gave IC_{50} values of 0.03–0.06 μM as compared with 0.002 μM for MTX.

In assays using L1210 cells, the *n*-butylamide (VIII.182) and benzylamide (VIII.183) had been found to have IC_{50} values of 0.09 and 0.03 μM, respectively, while the IC_{50} of MTX was 0.01 μM [299]. The IC_{50} values for inhibition of DHFR from L1210 cells were 3.3 and 3.0 nM as compared with 2.9 nM for MTX. Thus, there was a greater disparity between the amides and parent acid with respect to cell growth inhibition than DHFR inhibition, in qualitative agreement with the results of Piper *et al.* [240]. Although this effect was ascribed to less efficient uptake of the amides than of MTX by the cells, an

additional factor whose importance was not appreciated at the time was that the γ-amides could not form polyglutamates.

When injected i.p. on a qd × 9 schedule into L1210 leukaemic mice, amides (VIII.182), (VIII.183), (VIII.188), (VIII.193) and (VIII.194) all produced a modest increase in survival (30–60% ILS) [240]. However, 50- to 200-fold higher doses of the amides than of the parent acid had to be administered in order to achieve a comparable therapeutic effect.

In a more recent study [316], the *in vitro* and *in vivo* activities of the AMT γ-amides (VIII.198)–(VIII.205) and the MTX γ-amides (VIII.196) and (VIII.197) were examined. The IC_{50} values of these compounds against purified DHFR from L1210 cells were lowest (0.04–0.05 μM) for the *t*-butylamide (VIII.198) and the 3,4-methylenedioxyanilides (VIII.196) and (VIII.213), and highest (0.1–0.2 μM) for the 1-adamantylamide (VIII.199) and the 3,4-dichlorobenzylamide (VIII.202). In general, however, the IC_{50} values of the amides were only 1.5- to 5-fold higher than those of the parent acids, suggesting that if the compounds were all taken up equally well across the cell membrane they would be approximately equiactive as inhibitors of cell growth. Not surprisingly, this did not prove to be the case, as the IC_{50} values of the amides against L1210 cells in fact spanned a 1000-fold range. The most potent members of this series against L1210 cells proved to be γ-(3,4-methylenedioxyanilides) (VIII.196) and (VIII.213) and the γ-anilide (VIII.204), whose IC_{50} values were approximately the same as that of AMT. Against human leukaemic lymphoblasts (CEM cells), on the other hand, (VIII.196) and (VIII.213) were substantially less active than AMT or MTX, suggesting that the human cells either took up these amides more slowly or were less efficient in cleaving the amide bond to form AMT. *In vivo* against L1210 leukaemia in mice, the γ-*t*-alkylamides (VIII.198) and (VIII.199) were inactive, but all the γ-*N*-aralkylamides and γ-arylamides of AMT showed a significant (> 50%) increase in lifespan at the optimal dose. The most effective analogue was the γ-(3,4-dichlorobenzylamide) (VIII.202), which produced a 110% ILS at 70 mg/kg (qd × 9) as compared with 122% for AMT at 0.5 mg/kg. The fact that this compound was the most active *in vivo* but among the least active in culture suggested that bioconversion to the parent acid was perhaps occurring in the tissues of the mouse (for example, the liver), rather than in the tumour. The position of aromatic substituents in the benzylamides influenced activity, as evidenced by the fact that the sterically hindered γ-(2,6-dichlorobenzylamide) (VIII.203) elicited only a 67% ILS at 100 mg/kg.

MTX γ-dimyristoylphosphatidylethanolamide (VIII.221) has been incorporated into liposomes with the intent of ultimately linking these 'MTX sensitized liposomes' to tumour-specific antibodies [335]. As part of the preliminary

work, the effect of the drug loaded liposomes themselves on cell growth and DHFR activity was determined. Incorporation of [^{3}H]dUrd into the DNA of 3T3 fibroblasts was decreased to 10% of controls in the presence of MTX-sensitized liposomes equivalent to 2 μM MTX. The same degree of DNA synthesis inhibition occurred at a free MTX concentration of 0.2 μM. A similar decrease in potency was observed for MTX-sensitized liposomes relative to MTX itself with regard to [^{3}H]dUrd incorporation in L1210 cells. Approximately the same 10-fold difference in potency was also observed between the water-soluble γ-glycerophosphatidylethanolamide derivative (VIII.222) and MTX. In addition, it was observed [329] that transport defective human leukaemic lymphoblasts (CEM/MTX) [297] were as sensitive as the parental line (CEM) to liposomes loaded with (VIII.221) but were cross-resistant to (VIII.222). As expected, liposomes loaded with MTX α-dimyristoylphosphatidylethanolamide or the MTX α,γ-bis(dimyristoylphosphatidylethanolamide) were less effective than those containing the γ-amide. The lack of cross-resistance to liposomes loaded with (VIII.221) by CEM/MTX cells suggested that this approach had the potential to partly overcome resistance based on transport. However, it was unclear whether there was any advantage over the use of simple lipophilic esters.

Biological data have been obtained for MTX γ-hydroxamic acid (VIII.223) [315] and MTX γ-hydrazide (VIII.224) [299]. The IC_{50} values of (VIII.223) against DHFR from L1210 cells (ligand binding assay) and from *L. casei* (spectrophotometric assay) were found to be 4.8 nM and 30 nM, respectively. The corresponding values for (VIII.224) were 12 nM and 13 nM. Thus the γ-NHOH group was more favourable for binding to the L1210 enzyme, whereas the γ-$NHNH_2$ group favoured binding to the *L. casei* enzyme. Moreover, while the binding of VIII.223 was enhanced relative to MTX toward the L1210 enzyme and decreased toward the *L. casei* enzyme, binding of (VIII.224) was diminished relative to MTX toward the L1210 enzyme and increased toward the *L. casei* enzyme. In assays against cultured L1210 and CEM cells, (VIII.223) had IC_{50} values of 0.062 μM and 0.25 μM, while the IC_{50} value of (VIII.224) against L1210 cells was 0.02 μM. Thus, (VIII.223) was more potent than (VIII.224) against L1210 cells in qualitative agreement with the DHFR inhibition data. *In vivo* against L1210 leukaemia in mice, (VIII.223) gave a 111% ILS at 40 mg/kg (q3d $\times$ 3), while MTX on the same schedule gave a 100% ILS at 60 mg/kg.

The relative abilities of MTX-F (VIII.217) and of MTX to inhibit DHFR and to be taken up into cells have been compared [317]. The K_i values of MTX-F as an inhibitor of DHFR from L1210 cells and from *L. casei* were found to be 62 and 45 nM, respectively, as compared with 41 and 15 nM for

MTX. Thus, introduction of the large lipophilic γ-substituent was only slightly detrimental to DHFR binding, confirming that the enzyme domain corresponding to the γ-terminal region of MTX has a high degree of 'bulk tolerance'. In a subsequent publication from the same laboratory [337] the K_D for dissociation of MTX-F from L1210 enzyme was reported to be 50 nM. However, interpretation of these results was complicated by the fact that DHFR appeared to form several different complexes with MTX-F, each of which might have had its own kinetics of binding and dissociation. Four well-resolved fluorescent bands were observed on gel electrophoresis of DHFR treated with MTX-F and NADPH.

In transport experiments, the K_i of MTX-F as an inhibitor of MTX was found to be 0.5 μM in L1210 cells and 1.6 μM in *L. casei*. The corresponding K_t values for MTX transport were 0.35 and 1.3 μM. A follow-up study [337] compared the kinetics of uptake of MTX and MTX-F in L1210/R8 cells, whose DHFR content was 100-fold elevated in comparison with wild-type L1210 cells, and whose ability to take up MTX was impaired. The K_t for MTX transport in the L1210/R8 cells was found to be 6.7 μM as compared with 0.9 μM in the L1210 cells, while maximal velocities for uptake were essentially the same. The K_i of MTX-F as an inhibitor of MTX uptake was 0.79 μM in the L1210/R8 cells as compared with 0.28 μM in the parent line. Thus, the affinity of MTX-F for the carrier protein was higher than that of MTX in both the sensitive and resistant cells. However, the question of whether MTX-F was taken up via the same transport mechanism as MTX was not unequivocally settled. Uptake by the cells was very slow, requiring several days of treatment at relatively high MTX-F concentrations to achieve saturation, and moreover, was not linear. The authors suggested that MTX-F might be binding to the MTX transport carrier protein without being transported across the cell membrane, and that uptake might occur in part by diffusion. It was also possible that MTX-F was binding to intracellular sites other than DHFR.

The interaction of MTX-F with DHFR and the kinetics of uptake of this compound into cells are of interest to compare with data obtained with PT430, a structurally similar fluoresceinated derivative of N^e-(4-amino-4-deoxy-N^{10}-methylpteroyl)-L-lysine which was synthesized and studied by Rosowsky *et al.* [326] (*vide infra*). A number of papers have described the use of MTX-F as a reporter ligand for DHFR in MTX-sensitive and MTX-resistant cell lines [319–325]. In general, it appears that MTX-F and PT430 are comparable except that the latter compound may be taken up more rapidly across the cell membrane and may have a lower rate of dissociation from DHFR once it is bound to the enzyme.

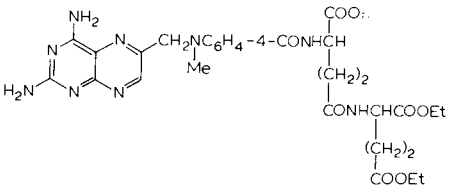
γ-Peptides and α,γ-dipeptides

Chemical synthesis

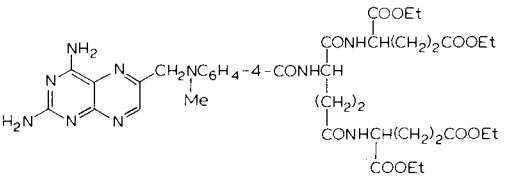
Derivatives of MTX in which the γ-carboxyl group is joined to an amino acid have been prepared in several laboratories, and are of interest in part because of the biological importance of the MTX γ-polyglutamates formed in cells by the enzyme FPGS. The role of polyglutamates of natural folates in cellular metabolism and of polyglutamates of MTX and other antifolates in blocking various enzymes of the folate pathway has been comprehensively reviewed [338, 339]. γ-Peptide derivatives of MTX are also of potential interest insofar as the peptide moiety can provide a biodegradable 'linker' between the parent drug and a synthetic macromolecular carrier [319] or tumour-specific monoclonal antibody [340].

The reaction of MTX with a small excess of diethyl L-glutamate in the presence of either diethyl phosphorochloridite or dicyclohexylcarbodiimide was investigated by Rosowsky and Yu [330] and was found to give a low yield of the γ-L-glutamate diethyl ester (VIII.229), along with the isomeric α-glutamyl diethyl ester and the corresponding α,γ-bis(L-glutamate tetraethyl ester) (VIII.231). In each case the major product was formulated as the α-peptide on the basis of its higher mobility on silica gel, and by comparison with an authentic sample obtained by a mixed anhydride condensation reaction between 4-amino-4-deoxy-N^{10}-methylpteroic acid (MeAPA) and diethyl α-L-glutamyl-L-glutamate. Purified yields of (VIII.229) were only 4.3% in the diethyl phosphorochloridite reaction and 11% in the DCC-catalyzed reaction as compared with yields of the α-isomer of 20% and 17%, respectively. It thus appeared that the α-carboxyl group in MTX was the more reactive site for peptide coupling reactions, perhaps because of its greater acidity. When the ratio of diethyl L-glutamate to MTX was increased, to 2.2:1, the α,γ-dipeptide became the dominant product. The same disubstituted compound could also be made by reaction of the α-peptide with a second molecule of diethyl L-glutamate in the presence of diphenylphosphoryl azide or by a mixed anhydride condensation reaction between MeAPA and tetraethyl α,γ-bis(L-glutamyl)-L-glutamate. Esterification of (VIII.229) with EtOH-HCl afforded the triethyl ester (VIII.230), which could also be prepared from MTX α-ethyl ester and diethyl L-glutamate in the presence of diphenylphosphoryl azide. An important feature of all the coupling reactions involving the α-carboxyl group was that the product appeared to consist of a mixture of diastereomers resulting from racemization of the α-carbon. A mechanism was proposed for this racemization, involving a non-chiral oxazolone intermediate. In the case of the α,γ-dipeptide, the two LLL and DLL diastereomers were actually isolated

and shown to have the same elemental composition by microanalysis. The discovery in this study that peptide bond-forming reagents induce racemization at the α-carbon of MTX was important because of the fact that D-MTX is substantially less active than L-MTX [164, 264] and that carbodiimide reagents have been used to couple MTX to macromolecules for such diverse purposes as the purification of dihydrofolate reductase on affinity columns [341], the production of anti-MTX antibodies for use in MTX radioimmunoassays [342], the conjugation of MTX to proteins and synthetic macromolecular carriers [343, 344], and the preparation of MTX-antibody conjugates for immunochemotherapy [345–349].

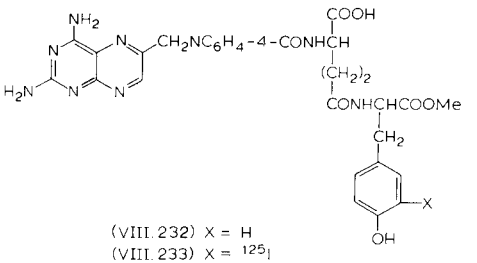


(VIII.229) R = H
(VIII.230) R = Et



(VIII.231)

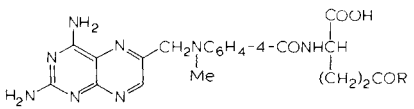
Condensation of MTX with tyrosine methyl ester in aqueous pyridine in the presence of EDC hydrochloride was used [350] to prepare the γ-peptide (VIII.232), along with the corresponding less polar α-peptide, from which it



(VIII.232) X = H
(VIII.233) X = ^{125}I

could be separated by preparative thin-layer chromatography. Radioiodination of the peptide yielded an ^{125}I derivative (VIII.233) suitable for use in radioimmunoassays based on competitive binding to anti-MTX antibody. The ester group in the tyrosine moiety was retained.

Structurally unequivocal γ-peptide derivatives of MTX have been prepared also from 2,4-diamino-6-bromomethylpteridine hydrobromide ((VIII.156) · HBr) [240]. Condensation of the bromide with unprotected *N*-[4-[*N*-(methylamino)benzoyl]-γ-L-glutamyl]-L-aspartic acid, and *N*-[4-[*N*-(methylamino)benzoyl]-γ-L-glutamyl)-L-glutamic acid in DMA at room temperature for days afforded (VIII.234) (72%), (VIII.235) (39%), and (VIII.236) (56%), respectively. In a subsequent study [351] this process was extended to the preparation of the MTX γ-oligoglutamates (VIII.237) (67%) and (VIII.238) (78%). The synthesis of the substituted *N*-[4-(*N*-methylamino)-benzoyl-L-glutamic acid derivatives used in the coupling reaction was straightforward. For example, condensation of dibenzyl L-glutamate with α-benzyl *N*-Boc-L-glutamate in the presence of diphenylphosphoryl azide, followed by acidolysis with HCl, condensation with dibenzyl *N*-[4-[*N*-Cbz-*N*-methylamino)benzoyl]-L-glutamate, and catalytic hydrogenolysis afforded *N*-[4-[*N*-Cbz-*N*-methylamino)benzoyl]-γ -L-glutamyl-γ-L-glutamyl-γ-L-glutamic acid.

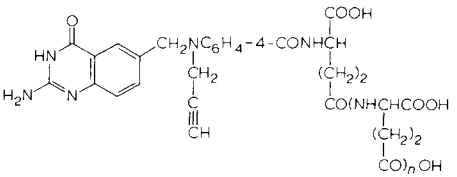


(VIII.234) R = $NHCH_2COOH$
(VIII.235) R = $NHCH(COOH)CH_2COOH$
(VIII.236) R = $NHCH(COOH)CH_2CH_2COOH$
(VIII.237) R = $NHCH(COOH)CH_2CH_2CONHCH(COOH)CH_2CH_2COOH$
(VIII.238) R = $[NHCH(COOH)CH_2CH_2CO]_2NHCH(COOH)CH_2CH_2COOH$
(VIII.239) R = $NH(CH_2)_3COOH$

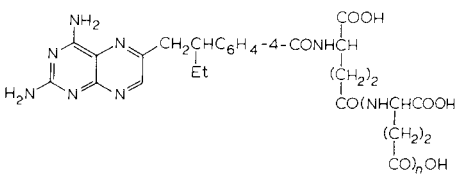
The MTX γ-peptide (VIII.239), in which the γ-carboxyl is joined to 4-aminobutyric acid, has been synthesized [352] as a de(carboxy) analogue of MTX + G_1. Condensation of α-benzyl L-glutamate with MeAPA by the diethyl phosphorocyanidate procedure gave a 94% yield of α-benzyl MTX, which on reaction with methyl 4-aminobutyrate in the presence of diphenylphosphoryl azide and subsequent ester hydrolysis with $Ba(OH)_2$ was converted to (VIII.239) in 50% overall yield.

In another example of the synthesis of γ-peptide derivatives of antifolates, Nair *et al.* [353] recently described a method of preparation of four γ-oligoglutamates of N^{10}-propargyl-5,8-dideazafolic acid (CB3717). N^{10}-Propargyl-5,8-

dideazapteroic acid was activated with isobutyl chloroformate and the mixed anhydride was added to γ-glutamyl esters of Merrifield resin. The α-COOH group of each glutamic acid was protected as a benzyl ester, and cleavage from the resin with concomitant hydrolysis of α-benzyl ester groups was accomplished with NaOH. γ-Oligoglutamates prepared in this manner included compounds (VIII.240)–(VIII.243), and yields ranged from 23 to 39% based on the amount of the dideazapteroic acid used. Similar procedures were also employed more recently [354] to obtain the four γ-oligoglutamates (VIII.244)–(VIII.247) of 10-ethyl-10-deazaAMT.



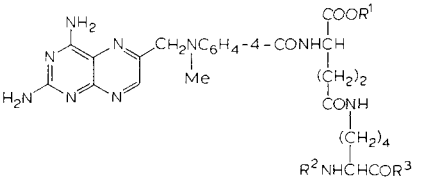
(VIII.240)-(VIII.243)(n = 1-4)



(VIII.244)-(VIII.247)(n = 1-4)

Peptide derivatives of MTX in which the γ-carboxyl group is linked to the ε-amino group of a lysine residue have also been described [355, 356]. Interest in these derivatives stemmed from the fact that they were putative lysosomal breakdown products of MTX-poly(L-lysine) [348, 357–361] and might have biological activity of their own. α-*t*-Butyl MTX was condensed with N^{α}-Boc-L-lysine methyl ester with the aid of diethyl phosphorocyanidate (DEPC) to obtain the diester (VIII.248) (76%) [348]. Hydrolysis of the methyl ester group with $Ba(OH)_2$ in aqueous ethanol then led to (VIII.249) in 87% yield, while further reaction of (VIII.249) with trifluoroacetic acid gave the diacid (VIII.250) [MTX(γ–ε)Lys_1] in 57% yield. Mixed anhydride condensation of α-benzyl MTX with the N^{ε},*O*-bis(trimethylsilyl) derivative of N^{α}-Boc-L-lysine afforded a 90% yield of the coupling product (VIII.251), which on further reaction with N^{α}-Cbz-L-lysine methyl ester in the presence of diphenylphosphoryl azide was converted to the blocked dilysyl derivative (VIII.252) (79%). Cleavage of the benzyl and methyl esters with $Ba(OH)_2$ afforded the diacid (VIII.253) (86%), while further treatment with trifluoroacetic acid in thioanisole gave the fully deprotected product (VIII.254) [MTX(γ–ε)Lys_2] in 77% yield. Removal of the Boc and Cbz groups with HBr-AcOH was also achieved, but in lower yield. Condensation of (VIII.251) with N^{α}-(N^{ε}-Cbz-L-lysyl)- N^{ε}-Cbz-L-lysine methyl ester in the presence of diphenylphosphoryl azide followed by hydrolysis first with $Ba(OH)_2$ and then with HBr-AcOH led sequentially

to compounds (VIII.255) (74%), (VIII.256) (94%) and (VIII.257) [MTX(γ–ε)Lys$_3$] (81%), respectively. Also prepared in a separate study [346] were the protected MTX(γ–ε)Lys$_2$ and MTX(γ–ε)Lys$_3$ derivatives (VIII.258)–(VIII.261), which were of interest because of the opportunity which the trimethylsilylethyl ester presented to carry out selective deprotection in the presence of a benzyl ester by reaction with tetrabutylammonium fluoride.

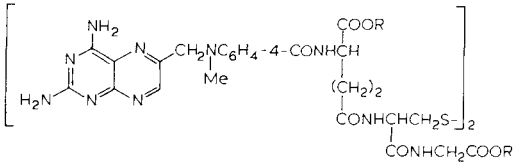


(VIII.248) R^1 = t-Bu, R^2 = Boc, R^3 = OMe
(VIII.249) R^1 = t-Bu, R^2 = Boc, R^3 = OH
(VIII.250) R^1 = R^2 = R^3 = OH
(VIII.251) R^1 = Bz, R^2 = Boc, R^3 = OH
(VIII.252) R^1 = Bz, R^2 = Boc, R^3 = NHCH[$(CH_2)_4$NHZ]COOMe
(VIII.253) R^1 = H, R^2 = Boc, R^3 = NH[$(CH_2)_4$NHCbz]COOH
(VIII.254) R^1 = R^2 = H, R^3 = NHCH[$(CH_2)_4NH_2$]COOH
(VIII.255) R^1 = Bz, R^2 = Boc, R^3 = NHCH[$(CH_2)_4$NHZ]CONH[$(CH_2)_4$NHZ]COOMe
(VIII.256) R^1 = H, R^2 = Boc, R^3 = NHCH[$(CH_2)_4$NHZ]CONH[$(CH_2)_4$NHZ]COOH
(VIII.257) R^1 = R^2 = H, R^3 = NHCH[$(CH_2)_4NH_2$]CONH[$(CH_2)_4NH_2$]COOH
(VIII.258) R^1 = Bz, R^2 = Boc, R^3 = NHCH[$(CH_2)_4$NHZ]COOR
(VIII.259) R^1 = Bz, R^2 = Boc, R^3 = NHCH[$(CH_2)_4$NHZ]COOH
(VIII.260) R^1 = Bz, R^2 = Boc, R^3 = NHCH[$(CH_2)_4$NHZ]CONHCH-[$(CH_2)_4$NHZ]COOR
(VIII.261) R^1 = Bz, R^2 = Boc, R^3 = NHCH[$(CH_2)_4$NHZ]CONHCH-[$(CH_2)_4$NHZ]COOH

(Bz = benzyl, Boc = t-butyloxycarbonyl, Z = benzyloxycarbonyl, R = 2-trimethylsilylethyl)

Another peptide derivative of MTX was prepared [362] with the aim of covalently attaching it to antibodies. Condensation of oxidized glutathione tetraethyl ester with 4-amino-4-deoxy-N^{10}-methylpteroic acid with the aid of diethyl phosphorocyanidate afforded the tetraethyl ester (VIII.262) (47% yield). Hydrolysis of the ester groups with $Ba(OH)_2$ followed by ion-exchange chromatography in the presence of 2-mercaptoethanol gave a crude product which was assumed to be the reduced tripeptide. On further purification by gel filtration without 2-mercaptoethanol, this compound underwent spontaneous oxidation to disulphide (VIII.263). In a model experiment, an IgG_1 monoclonal antibody directed against human transferrin receptor was activated by reaction with N-succinimidyl-3-(2-pyridyldithio)propionate, and con-

densed with the reduced form of (VIII.263), which was generated just before use by treatment with excess 2-mercaptoethanol. Spectrophotometric estimation of the amount of MTX in the conjugate led the authors to conclude that only one thiol group per antibody molecule had reacted.



(VIII.262) R = Et
(VIII.263) R = H

Biological activity

In vitro and *in vivo* antitumour data for the γ-peptide and α,γ-dipeptide esters (VIII.229)–(VIII.231) have been reported [330], but the results were of marginal interest. In growth inhibition assays against human leukaemic lymphoblasts (CEM cells), all three compounds had IC_{50} values greater than 1 μM. Not surprisingly, the DLL diastereomer of (VIII.231) was less active than the LLL diastereomer. In the *in vivo* assay against L1210 leukaemia in mice, (VIII.229) gave a 40% ILS at the maximally tolerated dose of 160 mg/kg (q3d × 3), while MTX gave a 60% ILS at 15 mg/kg.

Biological data for the MTX (γ–ε)lysine conjugates (VIII.250), (VIII.254) and (VIII.257) have also been reported [356]. While the IC_{50} value of MTX against purified DHFR from L1210 cells was 50 nM, the addition of either one lysine residue or two lysine residues at the γ-position gave an IC_{50} of 86 nM and the addition of a third lysine gave an IC_{50}value of 140 nM. Thus there was a 1.5- to 3-fold decline in binding affinity as the number of lysines increased from one to three. Moreover, the DHFR titration curves for the lysine derivatives showed a progressive increase in deviation from linearity, suggesting that positively charged groups on the ligand increased its rate of dissociation (the 'off-rate') from the enzyme-inhibitor complex. In growth inhibition assays against L1210 cells, the lysine derivatives were found to be 0.76, 1.8 and 2.9 μM, respectively. The addition of one lysine therefore caused a 31-fold reduction in potency relative to MTX (IC_{50} = 0.024 μM), while two and three lysines led to 77-fold and 120-fold decreases, respectively. The most likely explanation for the fact that the dilysine and trilysine derivatives were much less active than would be expected from their binding affinities to isolated DHFR was that the net positive charge on the side-chain of these compounds was detrimental to uptake across the cell membrane. Interestingly, the IC_{50}

values of the three lysine derivatives against H35 rat hepatoma cells were all in the 0.4–0.6 μM range, as compared with 0.01 μM for MTX. Thus, while activity against H35 cells in comparison with MTX again decreased on addition of lysine residues, these cells were apparently less sensitive than L1210 cells to differences in net charge on the side-chain. The lysine derivatives were also tested against a transport defective subline ($H35R_{0.3}$) and were found to be 15- to 20-fold less potent than MTX. The IC_{50} values of (VIII.250), (VIII.254) and (VIII.257) against the $H35R_{0.3}$ cells were in the 6–13 μM range as compared with 1.8 μM for MTX. Since the $H35R_{0.3}$ cells were 180-fold resistant to MTX, it appeared that MTX resistance had been partly overcome. Assays of the lysine derivatives against L1210 leukaemia in mice were carried out to test the possibility that the lysine derivatives might be bioactivated *in vivo* via cleavage of the N^{ε}-(γ-L-glutamyl)-L-lysine bond. Maximally tolerated doses of 40 mg/kg (bid × 10) afforded lifespan increases of 89% (VIII.250), 67% (VIII.254) and 55% (VIII.257), whereas MTX on the same schedule produced a 100% ILS at 0.5 mg/kg. In general these *in vivo* results were consistent with the *in vitro* data, and suggested that bioconversion to MTX, if it occurred at all, was minimal.

The biochemical and biological properties of the γ-peptides (VIII.234)–(VIII.236) have been compared [240], and appear to be in general accord with those of other γ-substituted MTX analogues. Thus, in assays of DHFR inhibition against enzyme from L1210 cells, the three peptides had K_i values of 2.9–3.7 pM as compared with 4.3 pM for MTX, and were *ca.* 10^5-fold more potent than the corresponding α-peptides. However, the potencies of the peptides against HEp.2 human epithelial carcinoma cells showed striking differences. While the γ-glycyl analogue (VIII.234) and γ-L-glutamyl analogue (VIII.236) had IC_{50} values of 5–6 nM and were therefore only 2-fold less active than MTX (IC_{50} = 2.4 nM) itself, the γ-L-aspartyl analogue (VIII.235) had an IC_{50} value of 330 nM and was therefore 150-fold less active than MTX. As expected, peptides (VIII.234) and (VIII.236) were up to 100-fold more active than their α-isomers. In contrast, (VIII.235) and its α-isomer had similar activity against the HEp.2 cells. The exact reason for the very low activity of the γ-L-aspartyl derivative is unclear, but is most likely related to inefficient transport and subsequent metabolism. In transport experiments using L1210 cells [240, 363], K_t values obtained for (VIII.234)–(VIII.236) were found to be 3.9, > 300 and 49 μM, respectively, whereas the K_t for MTX uptake was 3.3 μM. An important feature of (VIII.234) is that after being taken up by cells it would be unlikely to undergo polyglutamylation without first being cleaved to MTX. The finding that (VIII.234) and MTX had very similar K_t values was of interest, since (VIII.234) may viewed as a chain-leng-

thened MTX analogue with two extra atoms separating the carboxyl groups. When the data for (VIII.234) and (VIII.235) are considered together, it seems reasonable that the higher activity of the γ-glycyl derivative may reflect more rapid cleavage to MTX in the cell, followed by formation of MTX polyglutamates. The nature of the amino acid joined to the γ-position of MTX would be expected not only to influence the rate of hydrolysis of the γ-peptide bond, but also to determine what hydrolytic enzyme actually cleaves this bond.

Despite the low activity of the γ-L-aspartyl derivative (VIII.235) against cultured cells, the compound proved surprisingly effective in prolonging the lifespan of mice with L1210 leukaemia [240]. When given at a dose of 5.0 mg/kg (qd × 9), (VIII.234) and (VIII.235) both increased survival by 65–75%, while the γ-L-glutamyl derivative, (VIII.236), gave a 65% ILS at 2.5 mg/kg and MTX gave a 48% ILS at 1.0 mg/kg. The unexpected activity of (VIII.235) in this experiment suggested that enzymatic cleavage of the peptide bond to form MTX might be occurring in the mouse but not in L1210 cells in culture.

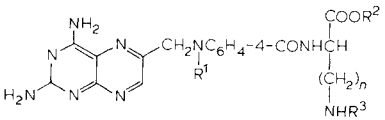
Novel use of (VIII.235) has been made in a study on liposome encapsulation of MTX [364]. A monoclonal antibody against murine L929 fibroblasts was chemically modified with *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) and covalently linked to 4-(*p*-maleimidophenyl)butyrylphosphatidylethanolamine. The resulting product was combined with phosphatidylcholine, cholesterol, and (VIII.235) to obtain liposomes, and the latter were compared with free drug for their ability to inhibit the growth of cultured L929 cells. Similar liposomes containing MTX in place of (VIII.235) were also prepared. The drug concentration inside the liposomes was determined spectrophotometrically to be 5 mM. The IC_{50} for inhibition of the growth of L929 cells incubated with (VIII.235) in targeted liposomes (*ca.* 5×10^4/cell) was 0.066 μM vs. 0.05 μM for MTX in targeted liposomes, 0.68 μM for (VIII.235) itself, and 0.018 μM for MTX. It thus appeared that encapsulation of (VIII.235) in a liposome successfully overcame the relatively inefficient uptake of this compound via the normal carrier-mediated pathway for MTX. Uptake of the targeted liposome presumably occurred via endocytosis, and was followed by lysosomal degradation since NH_4Cl blocked the growth-inhibitory effect of encapsulated drug but not of free MTX. The fact that encapsulated (VIII.235) and encapsulated MTX had comparable molar potencies suggested that the γ-aspartyl derivative was being degraded to MTX in the lysosomes.

REPLACEMENT OF GLUTAMIC ACID BY α, ω-DIAMINO MONOACIDS

Chemical synthesis

The first reported example of an MTX analogue containing a basic amino-acid side-chain was the lysine derivative (VIII.264), which was prepared in low yield [196] from 2,4,5,6-tetraaminopyrimidine, 2,3-dibromopropionaldehyde, and methyl N^{ε}-Cbz-N^{α}-[4-(*N*-methylaminobenzoyl]-L-lysinate via a Waller synthesis. After hydrolysis of ester (VIII.265) with HBr, ion-exchange chromatographic purification gave (VIII.264) in only 6.5% overall yield. Subsequently an improved synthesis of (VIII.264) was described [365], in which 4-amino-4-deoxy-N^{10}-methylpteroic acid was condensed in 91% yield with methyl N^{ε}-Boc-N^{α}-[4-(*N*- methylamino)benzoyl]-L-lysinate with the aid of the peptide bond-forming reagent, diethyl phosphorocyanidate (DEPC), the resulting methyl ester (VIII.266) was cleaved with aqueous piperidine (84% yield), and the Boc group was removed with boron tribromide (78% yield). Treatment of (VIII.266) with trifluoroacetic acid afforded (VIII.264) directly (78% yield). In a variant of this approach [357, 358], 4-amino-4-deoxy-N^{10}-methylpteroic acid was condensed with *t*-butyl N^{ε}-Boc-N^{α}-[4-(*N*-methylamino)benzoyl]-L-lysinate and the Boc and *t*-butyl ester groups in the coupling product (VIII.267) were removed with trifluoroacetic acid.

Lower homologues of (VIII.264) have likewise been prepared in the form of the L-ornithine analogue (VIII.268), the 2,4-diaminobutyric acid analogue (VIII.269), and the 2,3-diaminopropionic acid analogue (VIII.270). In one synthetic approach [368], 2,4-diamino-6-bromomethylpteridine was condensed with ethyl N^{γ}-Boc-N^{α}-[4-(*N*-methylamino)benzoyl]-L-2,4-diaminobut-



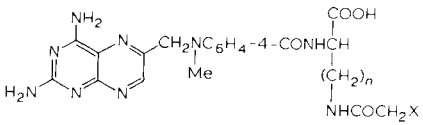
(VIII.264) R^1 = Me, R^2 = R^3 = H, n = 4
(VIII.265) R^1 = R^2 = Me, R^3 = Cbz n = 4
(VIII.266) R^1 = R^2 = Me, R^3 = Boc, n = 4
(VIII.267) R^1 = Me, R^2 = *t*-Bu, R^3 = Boc, n = 4
(VIII.268) R^1 = Me, R^2 = R^3 = H, n = 3
(VIII.269) R^1 = Me, R^2 = R^3 = H, n = 2
(VIII.270) R^1 = Me, R^2 = R^3 = H, n = 1
(VIII.271) R^1 = Me, R^2 = Et, R^3 = Boc, n = 2
(VIII.272) R^1 = Me, R^2 = Et, R^3 = Boc, n = 1
(VIII.273) R^1 = CHO, R^2 = H, R^3 = Boc, n = 3
(VIII.274) R^1 = R^2 = H, R^3 = Boc, n = 3
(VIII.275) R^1 = R^2 = R^3 = H, n = 3

anoate and ethyl N^{β}-Boc-*N*-[4-(*N*-methylamino) benzoyl]-L-2,3-diaminopropanoate to obtain the ethyl esters (VIII.271) (79%) and (VIII.272) (79%), respectively. Hydrolysis of the ester groups with NaOH (> 90% yield) and of the Boc group with trifluoroacetic acid (> 90% yield) afforded the deprotected products (VIII.269) and (VIII.270). For the preparation of (VIII.268), the coupling reaction was performed with N^{δ}-Boc-N^{α}-[4-(*N*-methylamino)-benzoyl]-L-2,5-diaminopentanoic acid (95% yield), thus avoiding the need for ester hydrolysis prior to Boc cleavage with trifluoroacetic acid or HCl.

In an independently developed synthesis of (VIII.268)–(VIII.270), 4-amino-4-deoxy-N^{10}-methylpteroic acid was condensed with N^{ω}-Boc derivatives of L-2,3-diaminopropanoic, L-2,4-diaminobutanoic and L-2,5-diaminopentanoic acids, and Boc groups were removed with trifluoroacetic acid [369]. The yield in the DEPC coupling reaction was good for the butanoic (87%) and pentanoic (72%) acid derivatives, but lower (34%) for the propanoic acid derivative, perhaps as a result of ring closure of N^{β}-Boc-L-2,3-diaminopropanoic acid to an imidazolidin-2-one under the conditions of the DEPC coupling reaction. An alternative route to (VIII.268) and (VIII.270) was also found in the reaction of the appropriate N^{ω}-Boc derivatives with *p*-nitrophenyl 4-amino-4-deoxy-N^{10}-methylpteroate, which was obtained in 86% yield from the acid by reaction with bis(*p*-nitrophenyl)carbonate and triethylamine in DMF at 55 °C. When 4-amino-4-deoxy-N^{10}-methylpteroic acid was replaced by 4-amino-4-deoxy-N^{10}-formylpteroic acid in this sequence and N^{δ}-Boc-L-ornithine was protected by N^{α},*O*-bis(trimethylsilylation), the coupling product was (VIII.273) (49%). Cleavage of the N^{10}-formyl group with base gave (VIII.274) (83%), and subsequent removal of the Boc group from (VIII.274) with trifluoroacetic acid yielded the amine (VIII.275) (89%).

The availability of MTX analogues with an animo group at the end of the side-chain provided an obvious opportunity to introduce into the molecule a variety of additional groups of potential theoretical and practical interest. Among the first in this regard were *N*-haloacyl derivatives, which had the potential to serve as irreversible active-site-directed inhibitors of dihydrofolate reductase. An early example of such a compound was N^{α}-(4-amino-4-deoxy-N^{10}-methylpteroyl)-N^{ε}-iodoacetyl-L-lysine (VIII.276), which was obtained from (VIII.264) in 32% yield on treatment with *N*-(iodoacetoxy)succinimide in MeOH at 55–60 °C (45 min) followed by recrystallization [365]. Subsequently, the *N*-bromoacetyl and *N*-chloroacetyl analogues (VIII.277) and (VIII.278) were prepared in 30–40% yield by reaction of (VIII.264) in aqueous $NaHCO_3$ at 50 °C with *p*-nitrophenyl bromoacetate and *p*-nitrophenyl chloroacetate, respectively [369]. Similarly prepared from the ornithine analogue (VIII.268) were the *N*-bromoacetyl and *N*-chloroacetyl derivatives

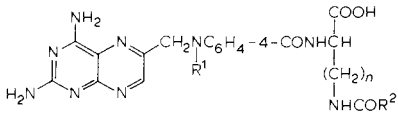
(VIII.279) and (VIII.280). After considerable experimentation, the optimal ratio of reactants was found to be 1:4:4 amine-$NaHCO_3$-acylating agent and the optimal length of reaction was found to be 2–2.5 h. Any deviation from these conditions caused significant decreases in yield. Purification was made difficult by the apparent tendency of the halohacyl derivatives to undergo not only simple solvolysis, but also polymerization via self-alkylation. The only successful method of purification found was chromatography in water on a column of Bio-Gel P2, a polyacrylamide size-exclusion resin with a fractionation range of 100–1800 Da. Product obtained in this manner was TLC-homogeneous and gave a strong positive test for alkylating activity with *p*-nitrobenzylpyridine. Acylations were also carried out with (VIII.269) and (VIII.270), but attempts to obtain the haloacylated products in pure state were unsuccessful due to rapid solvolysis and self-alkylation. In general, stability, and therefore ease of isolation, seemed to depend on the number of CH_2 groups ($1 < 2 < 3 < 4$) and, not surprisingly, on the reactivity of the halogen substituent ($I < Br < Cl$).



(VIII.276) X = I, $n = 4$
(VIII.277) X = Br, $n = 4$
(VIII.278) X = Cl, $n = 4$
(VIII.279) X = Br, $n = 3$
(VIII.280) X = Cl, $n = 3$

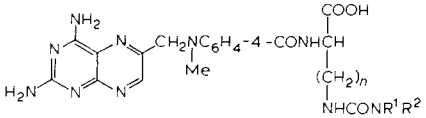
Piper *et al.* [368] acylated the terminal amino group in the MTX analogues (VIII.268)–(VIII.270) with 4-chlorobenzoyl chloride to form the amides (VIII.281)–(VIII.283). More recently a series of N^{δ}-acyl derivatives of the AMT analogue (VIII.275) were prepared from N^{α}-(4-amino-4-deoxy-N^{10}-formylpteroyl-L-ornithine (VIII.274) by reaction with appropriate anhydrides to give the N^{10}, N^{δ}-disubstituted derivatives (VIII.284)–(VIII.289) [370]. Selective hydrolysis at N^{10} with dilute NaOH then gave the N^{δ}-acyl derivatives (VIII.290)–(VIII.295). The impetus behind these studies was a desire to improve cellular penetration, which is hindered in the parent compounds by protonation of the side-chain amino group at physiologic pH.

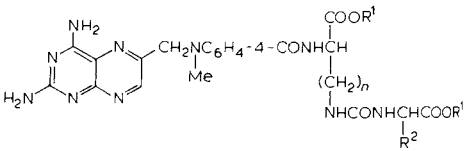
Another class of acylated derivatives reported by Piper *et al.* [368] were the ureas (VIII.296)–(VIII.298), which were obtained from 2,4-diamino-6-bromomethylpteridine by reaction with N^{ω}-carbamoyl and N^{ω}-methylcarbamoyl



(VIII.281) R^1 = Me, R^2 = C_6H_4(4-Cl), n = 3
(VIII.282) R^1 = Me, R^2 = C_6H_4(4-Cl), n = 2
(VIII.283) R^1 = Me, R^2 = C_6H_4(4-Cl), n = 1
(VIII.284) R^1 = CHO, R^2 = Me, n = 3
(VIII.285) R^1 = CHO, R^2 = CH_2CH_2COOH, n = 3
(VIII.286) R^1 = CHO, R^2 = C_6H_5, n = 3
(VIII.287) R^1 = CHO, R^2 = C_6H_4(4-Cl), n = 3
(VIII.288) R^1 = CHO, R^2 = C_6H_3(3,4-Cl_2), n = 3
(VIII.289) R^1 = CHO, R^2 = C_6H_4(2-COOH), n = 3
(VIII.290) R^1 = H, R^2 = Me, n = 3
(VIII.291) R^1 = H, R^2 = CH_2CH_2COOH, n = 3
(VIII.292) R^1 = H, R^2 = C_6H_5, n = 3
(VIII.293) R^1 = H, R^2 = C_6H_4(4-Cl), n = 3
(VIII.294) R^1 = H, R^2 = C_6H_3(3,4-Cl_2), n = 3
(VIII.295) R^1 = H, R^2 = C_6H_4(2-COOH), n = 3

derivatives of N^{α}-[4-(*N*-methylamino)benzoyl]-L-2,3-diaminopropanoic and N^{α}-[4-(*N*-methylamino)benzoyl]-L-2,4-diaminobutanoic acids. Further reaction of (VIII.296) with 2,4,5-trichlorophenyl *N*-methyl-*N*-nitrosocarbamate and 2,4,5-trichlorophenyl *N*-(2-chloroethyl)-*N*-nitrosocarbamate yielded the interesting nitrosourea analogues (VIII.299) (84% yield) and (VIII.300) (74% yield), respectively. As with the *N*-haloacetyl derivatives, there was the possibility in principle that these nitrosoureas could act as active-site-directed irreversible inhibitors of DHFR.





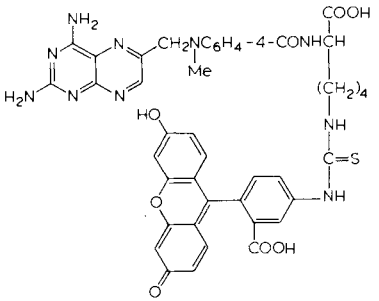
(VIII.296) R^1 = R^2 = H, n = 1
(VIII.297) R^1 = R^2 = H, n = 2
(VIII.298) R^1 = H, R^2 = Me, n = 1
(VIII.299) R^1 = Me, R^2 = NO, n = 1
(VIII.300) R^1 = CH_2CH_2Cl, R^2 = NO, n = 1

(VIII.301) R^1 = Et, R^2 = H, n = 1
(VIII.302) R^1 = Et, R^2 = H, n = 2
(VIII.303) R^1 = Et, R^2 = CH_2CH_2COOEt, n = 1
(VIII.304) R^1 = Et, R^2 = CH_2CH_2COOEt, n = 2
(VIII.305) R^1 = H, R^2 = CH_2CH_2COOH, n = 1

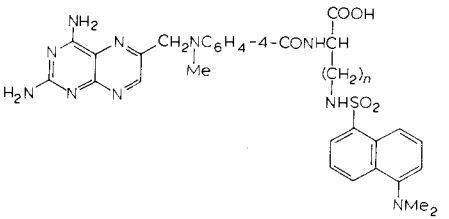
Further examples of the ability to elaborate the terminal amino group in (VIII.296) and (VIII.297) were provided [368] in the reaction of these compounds with ethyl isocyanotoacetate, which gave the glycyl derivatives (VIII.301) (45%) and (VIII.302) (61%), and with diethyl 2-isocyanatoglutarate, which gave the glutamyl derivatives (VIII.303) (72%) and (VIII.304)

(79%). Attempted hydrolysis of the ester groups with NaOH resulted in unwanted ring closure reactions (possibly to hydantoins in the case of (VIII.301) and (VIII.302) and pyroglutamyl derivatives in the case of (VIII.303) and (VIII.304); however, hyrdolysis of the propanoic acid derivative (VIII.303) with $Ba(OH)_2$ instead of NaOH gave a 46% yield of the interesting MTX diglutamate analogue, (VIII.305).

Reaction of the lysine derivative (VIII.264) with fluorescein isothiocyanate was described in 1981 by Rosowsky *et al.* [326]. The resulting product (PT430, (VIII.306)) was used in a number of experiments designed to take advantage of its ability to serve as a fluorescent DHFR ligand in intact cells. The presence of a fluorescein moiety in PT430 made this compound especially useful for flow cytometry [326, 371]. Excitation and emission wavelengths were at 488 and 518 nm, respectively. Fluorescent compounds have been prepared also by reaction of (VIII.264) or the ornithine analogue (VIII.268) with dansyl chloride [366, 373–375]. However the fluorescence properties of the resulting products (VIII.307) and (VIII.308) (excitation at 328 nm, emission at 580 nm) are less useful for flow cytometry than those of PT430. It should be noted that PT430 resembles 'MTX-F', the product formed by reaction of *N*-[N^{α}-(4-amino-4-deoxy-N^{10}-methylpteroyl)-L-γ-glutamyl]-1,5-pentanediamine with fluorescein isothiocyanate [317, 318] (*vide supra*). However, two structural differences between PT430 and 'MTX-F' are worth noting: first, the absence of a 1,5-pentanediamine 'spacer' in PT430 means that the molecule is a little smaller and perhaps diffuses more easily into cells; second, the fact that the amino acid moiety is lysine rather than glutamic acid means that no free MTX can be formed chemically or enzymatically in cells or cell culture medium.



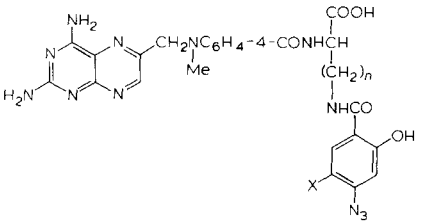
(VIII.306)



(VIII.307) $n = 4$
(VIII.308) $n = 3$

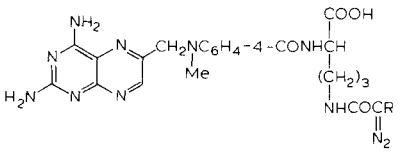
Other interesting probes of the active-site folate transport and binding have been prepared from (VIII.264) and (VIII.268) by reaction with *N*-hydroxy-

succinimidyl-4-azidosalicylate [376]. The resulting amides (VIII.309) and (VIII.310) could be treated with excess $Na^{125}I$ and chloramine T to form the radioactive photoaffinity reagents (VIII.311) and (VIII.312), respectively [377]. These reagents proved very useful in studies of the active-site binding region in DHFR from L1210 cells [378], as well as in studies directed toward the isolation, purification and sequencing of the membrane carrier protein for MTX in L1210 cells [379].

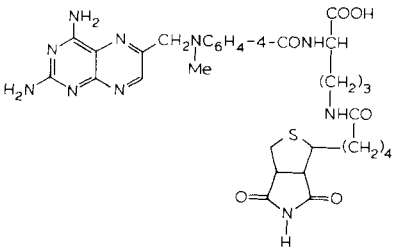


(VIII.309) $n = 4$, X = H
(VIII.310) $n = 3$, X = H
(VIII.311) $n = 4$, X = I
(VIII.312) $n = 3$, X = I

Other novel biochemical probes prepared from the ornithine analogue (VIII.268) were the diazo compounds (VIII.313) and (VIII.314) [376], and the biotin derivative (VIII.315) [376]. Another interesting biotin derivative (VIII.228) (*vide supra*) has been prepared from α-*t*-butylMTX by condensation with biotin hydrazide followed by acidolysis [333].



(VIII.313) R = COOEt
(VIII.314) R = CF_3



(VIII.315)

Biological activity

Compound (VIII.264), the lysine analogue of MTX, was tested as an inhibitor of murine and human DHFR [196] and found to have IC_{50} values of 5 and 3 nM, respectively, against these two enzymes. It was evident, however, that

introduction of an amino group at the end of the amino-acid side-chain was very detrimental to biological activity. Although cell culture data were not reported, *in vivo* experiments in L1210 leukaemic mice showed that a single dose of 400 mg/kg was well tolerated but gave no increase in survival whatsoever. More recently, Rosowsky *et al.* [365] reported that in a competitive radioligand binding assay using a non-purified DHFR preparation from L1210 cells the IC_{50} value of (VIII.264) was 11 nM, whereas the IC_{50} against *L. casei* was 122 nM. The IC_{50} values for MTX standards in the same assays were 3 and 16 nM. Thus, it appeared that introduction of a basic group resulted in decreased binding, and that this change was greater for the bacterial than for the mammalian enzyme. In a subsequent study in which DHFR inhibition was determined with purified enzyme by spectrophotometric (functional) rather than radioligand binding (non-functional) assay, (VIII.264) and MTX gave IC_{50} values of 65 and 35 nM, respectively [369]. There was thus a 2-fold decrease in binding on replacement of the CH_2CH_2COOH moiety in Region G of MTX by $CH_2CH_2CH_2CH_2NH_2$. Interestingly, there was a further 2- to 3-fold decrease in binding for the shorter-chain analogues (VIII.268) (n = 3, IC_{50} = 160 nM), (VIII.269) (n = 2, IC_{50} = 120 nM), and (VIII.270) (n = 1, IC_{50} = 180 nM). The IC_{50} of the AMT analogue (VIII.275) was 72 nM as compared with 35 nM for AMT, showing again a 2-fold decrease in binding on replacement of CH_2CH_2COOH by $CH_2CH_2CH_2$-CH_2NH_2. Inhibition of DHFR by (VIII.264) and (VIII.268) has also been measured spectrophotometrically in a functional assay [366]. The IC_{50} values were found to be 13 and 25 nM, respectively, as compared with 9 nM for MTX. The N^{ω}-Cbz derivatives was roughly half as inhibitory as the parent amines, while esterification of the α-carboxyl group, as expected, led to more substantial loss of binding.

If DHFR inhibition were the only determinant of the ability of a compound to inhibit cell growth, (VIII.264) and (VIII.268)–(VIII.270) would be expected not to differ in potency from MTX by more than an order of magnitude. In assays against L1210 murine leukaemia cells [369], however, the IC_{50} values for these compounds ranged from 0.4 μM ((VIII.264)) to 2.4 μM ((VIII.269)), as compared with 0.002 μM for MTX. For (VIII.275) the IC_{50} value was 1.3 μM as compared with 0.002 μM for AMT. Thus, growth inhibitory potency was decreased 200- to 1200-fold on replacement of glutamate by basic amino acids, even though DHFR affinity was decreased < 10-fold. The most probable reason for this discrepancy is that introduction of a basic amino group was detrimental to penetration of the cell membrane. Consistent with this idea was the fact that when these compounds were tested against L1210/R81 cells, which are > 10^5-fold resistant to MTX by virtue of a

profound defect in active transport [49], their IC_{50} values (80–400 μM) differed only slightly from the IC_{50} value of MTX (220 μM). Cross-resistance between (VIII.275) and AMT was likewise essentially complete.

An important property of the ornithine analogues (VIII.268) and (VIII.275) of MTX and AMT, respectively, was found to be their ability to inhibit folylpolyglutamate synthetase (FPGS) [369, 380]. Against partially purified FPGS from mouse liver [369], the MTX analogue (VIII.268) gave a K_i of 20.4 $\pm$ 7.7 μM with folic acid as the variable substrate, while for the AMT analogue, (VIII.275), a substantially lower K_i of 0.15 $\pm$ 0.06 μM was observed. Inhibition of FPGS was exquisitely sensitive to the length of the side-chain, as the analogues with n = 1,2, and 4 all gave $< 10\%$ inhibition of polyglutamylation when equal 500 μM concentrations of inhibitor and folic acid substrate were used. It was proposed that the remarkable specificity of the ornithine analogues reflected the fact that the ω-amino group in these compounds is at just the right distance from the α-carbon to be able to interact with a putative acidic residue in the active site whose normal role is to form an ionic bond to the *α-amino group of the incoming glutamic acid* [287]. In this sense, these molecules may therefore be viewed as inhibitors of the binding of both the folate substrate and the glutamate substrate. In a more recent publication [288], the abilities of the ω-amino analogues of MTX and AMT to competitively inhibit partially purified FPGS from human liver were also compared. With AMT as the variable substrate, estimated K_i values of 165 $\pm$ 36 (VIII.270), 690 $\pm$ 80 (VIII.269), 13.4 $\pm$ 0.1 (VIII.268) and 0.23 $\pm$ 0.01 μM (VIII.275) were obtained. K_i values determined with folic acid, MTX and MTX diglutamate ($MTX + G_1$) as the variable substrate were all in the 0.1–0.3 μM range. The finding that (VIII.275) was equally inhibitory with MTX or $MTX + G_1$ as the substrate was of particular importance, since it suggested that this compound would inhibit both the first and second step of polyglutamate synthesis. Inhibition of FPGS partially purified from a human colon carcinoma cell line (VRC-5) grown in immunosuppressed mice has also been examined with (VIII.268) and (VIII.275) [289]. The potency of these compounds appeared to be several times greater against the human tumour enzyme than against the mouse liver enzyme. Since similar K_i values have been obtained for inhibition of human and mouse liver FPGS by (VIII.275) [288, 369], the results with human colon carcinoma FPGS suggest that it may be possible to achieve inhibition of polyglutamylation in tumours.

The biochemical pharmacology of the MTX analogues (VIII.268)–(VIII.270) has been investigated also by McGuire *et al.* [380]. In assays using purified DHFR from K562 human myeloblastic leukaemia cells and CCRF-CEM lymphoblastic leukaemia cells, IC_{50} values of 2–4 nM for

(VIII.268) ($n = 3$), 5–8 nM for (VIII.269) ($n = 2$), and 17–18 nM for (VIII.270) ($n = 1$) were obtained. By comparison, the IC_{50} value for MTX was 1 nM. There was thus a progressive decrease in binding as more CH_2 groups were added. The difference in IC_{50} value between the ω-amino compounds and MTX in these assays ranged from 2- to 20-fold, whereas in the L1210 assay it had been reported to be 2- to 5-fold [369]. In assays of growth inhibitory activity against K562 cells, the amino compounds gave IC_{50} values of 1.7–3.0 μM in comparison with 0.18 μM for MTX. Against CCRF-CEM cells, the ornithine analogue (VIII.268) gave an IC_{50} value of 0.74 μM. Hypoxanthine (10 μM) alone and thymidine (10 μM) alone afforded only 10% protection of K562 cells from an IC_{90} dose of (VIII.268), while the combination of the two gave 30% protection and leucovorin (0.05 μM) was completely protective.

The ability of the amino compounds (VIII.268)–(VIII.270) to competitively inhibit FPGS from rat liver and human leukaemic cells (lines K562 and CCRF-CEM) was also evaluated [380]. As reported for FPGS from mouse liver [369] and human liver [288], only the ornithine analogue (VIII.268) was a potent inhibitor, with a K_i of 3–4 μM as compared with 100–200 μM for the diaminobutanoic acid analogue (VIII.269) and > 1000 μM for the diaminopropanoic acid analogue (VIII.270). Co-incubation of rat liver FPGS with MTX (10 μM) and L-[^{3}H]glutamate (4 mM) in the presence of (VIII.268) (5 μM), MTX + G_1 formation was inhibited by 57% while MTX + G_2 formation was inhibited by 76% and MTX + G_3 formation was undetectable. Similarly, when CCRF-CEM cells were incubated for 4 h in the presence of 10 μM [^{3}H]MTX and 50 μM (VIII.268), the formation of all the polyglutamates from G_2 to G_4 was decreased by 40–50%; the synthesis of MTX + G_5 was also inhibited, but to a lesser extent. However, since unchanged MTX was also decreased by 40–50% and total drug was decreased by 25%, it was difficult to be sure that (VIII.268) was actually interfering with the action of FPGS inside the cell. Moreover, when extracellular MTX was removed and non-glutamylated intracellular MTX was allowed to efflux, inhibition of MTX polyglutamate elongation was minimal. These results made it unlikely that FPGS inhibition could contribute to the effect of (VIII.268) on cell growth unless a much higher concentration of drug could be achieved in the cell.

The effect of N^{ω}-(4-chlorobenzoylation) in MTX analogues (VIII.281)–(VIII.283) was examined by Piper *et al.* [368]. The apparent K_i against purified DHFR from L1210 cells ranged from 5.0 to 6.4 pM as compared with 5.8 pM for MTX, showing that substitution of a bulky hydrophobic group for the terminal amino group is a well-tolerated structural modification as far as DHFR binding is concerned. In assays against L1210 cells in culture, on the

other hand, compounds (VIII.283) (n = 1), (VIII.282) (n = 2) and (VIII.281) (n = 3) gave IC_{50} values of 0.012, 0.027 and 0.0017 μM, respectively, as compared with 0.0033 μM for MTX. There was thus a relationship between the number of CH_2 groups and the ability of the analogues to inhibit cell growth. The IC_{50} values observed for the parent amines (VIII.270) (n = 1), (VIII.269) (n = 2) and (VIII.268) (n = 3) were 1.0, 1.3 and 0.51 μM, respectively. Thus, N^{ω}-(4-chlorobenzoylation) brought about increases in growth inhibitory potency of 40- to 300-fold, depending on the baseline activity of the parent amine. Compound (VIII.281) (n = 3) was approximately twice as potent as MTX.

The K_i for inhibition of unidirectional [^{3}H]MTX influx into L1210 cells by compounds (VIII.281)–(VIII.283) was examined [368] and found to range from 4.3 to 7.3 μM, versus 4.6 μM for MTX. The efflux rate constant was also examined and found to range from 0.26 to 1.2 min^{-1}, versus 0.20 min^{-1} for MTX. However, there appeared to be no clear correlation between these transport parameters and growth inhibitory activity. Since the K_i of (VIII.281) as an inhibitor of unidirectional [^{3}H]MTX influx into L1210 cells was about the same as that of unlabeled MTX, its potency in cell culture was not due merely to a higher rate of active transport. It was possible, on the other hand, that the potency of (VIII.281) was influenced by diffusion, which would be favoured by the lipophilicity of the molecule relative to MTX. Given the likelihood that this compound and its homologues accumulate in cells via a combination of diffusion and active transport, rigorous kinetic correlations based on competitive transport experiments would be expected to be difficult.

In vivo testing of the antitumour activity of (VIII.281) was performed in L1210 leukaemic mice [368]. While MTX at 12 mg/kg (q2d × 5) prolonged survival by 173%, a maximally tolerated dose (VIII.281) (768 mg/kg) on the same schedule gave an ILS of 104% ILS. Thus, (VIII.281) did not produce the same therapeutic effect as MTX, even when administered at a 60-fold higher dose.

In vitro and *in vivo* studies have been reported for the ureas (VIII.296)–(VIII.298) and the nitrosoureas (VIII.299) and (VIII.300) [368]. The K_i values of the ureas (VIII.296)–(VIII.298) as inhibitors of DHFR from L1210 cells were found to 35, 0.0004, and 14 nM, respectively, whereas the K_i of MTX was 0.006 nM. Thus, there was a large difference in binding, depending on whether one or two CH_2 groups were present in the side-chain, but a much smaller difference between compound with N^{ω}-carbamoyl and N^{ω}-methylcarbamoyl substitution on the terminal nitrogen. The ureidopropanoic acid analogue (VIII.296) and MTX were nearly equipotent in this system. The *N*-methylnitrosourea (VIII.299) and N^{ω}-(2-chloroethyl)nitroso-

urea (VIII.300) had K_i values of 64 and 32 nM, and were therefore comparable in binding to non-nitrosated urea derivatives of the same chain length. No evidence of time-dependent covalent binding to DHFR by the chloroethyl derivative (VIII.300) was reported. In assays of transport into cultured cells, the ureas (VIII.296)–(VIII.298) gave results suggesting selectivity for tumour versus normal epithelial cells. The K_i for inhibition of [^{3}H]MTX unidirectional influx was in the 10–25 μM range, whereas the corresponding value for epithelial cells was $> 500\,\mu$M. The K_i(epithelial)/K_i(tumour) was therefore greater than 20:1, which would be expected to be translated into a favourable therapeutic index *in vivo*. Against L1210 cells in culture, the IC_{50} values for compounds (VIII.296)–(VIII.298) were found to be 0.0094, 0.022 and 0.0344 μM, as compared with 0.0033 μM for MTX. Thus, the correlation between DHFR inhibition and cell growth inhibition was very poor. For example, (VIII.296) had a 6000-fold lower affinity than MTX for purified enzyme (as well as a lower affinity for the MTX/reduced folate transport pathway) but was nearly equipotent with MTX in culture. Moreover, in assays against L1210 leukaemia in mice, the propanoic acid derivative (VIII.296) (33% ILS at 432 mg/kg, q2d × 5) was far less effective than the butanoic acid derivative (VIII.297) (129% ILS on the same dose schedule), which was only slightly less potent in culture. At present there is no obvious explanation for these interesting aberrant results.

The K_i values of the nitrosoureas (VIII.299) and (VIII.300) as competitive inhibitors of [^{3}H]MTX transport were 40–50 μM [368] and were therefore only a little higher than those of the non-nitrosated ureas. The IC_{50} values of these compounds against cultured L1210 cells, however, were 20–40 μM and were therefore about 1000-fold higher than those of the parent ureas, even though potency against purified DHFR was similar (*vide supra*). It is difficult to reconcile these results unless one assumes that when the nitrosoureas are incubated in the culture medium they form inactive breakdown products or are converted to products that bind to serum proteins. *In vivo* against P388 murine leukaemia on a qd × 5 schedule, (VIII.299) gave a 58% ILS at 200 mg/kg, while (VIII.300) gave a 113% ILS at 400 mg/kg. However, data against L1210 leukaemia were not reported. It is not clear how much of the activity observed with (VIII.300) is due to an antifolate effect and how much, if any, is due to alkylation of DNA by chloroethyl carbonium ion or other reactive intermediates. Whether or not this type of 'two-fisted' agent has therapeutic potential remains unproven at this time.

In addition to the other studies summarized above, Piper *et al.* [368] examined the biological activity of the MTX diglutamate analogue (VIII.305), and found the compound to be a good inhibitor of DHFR (K_i = 0.0047 nM

versus 0.0058 nM for MTX) but not a very efficient competitive inhibitor of [^{3}H]MTX influx (K_i = 16 μM versus 4.6 μM for MTX). Furthermore, (VIII.305) was relatively inactive as an inhibitor of L1210 cell growth, with an IC_{50} of 0.2 μM as compared with 0.0033 μM for MTX. It would appear that the addition of a third carboxyl group retarded, but did not completely abrogate, uptake into L1210 cells. Activity *in vivo* was not reported.

Rosowsky *et al.* [371] recently found that some of the *N*-acyl derivatives of the AMT analogue (VIII.275) are exceptionally potent against cultured cells and, perhaps more importantly, have excellent activity against cells with low level (20- to 250-fold) resistance to MTX. In assays against purified DHFR from L1210 cells, the amides (VIII.290)–(VIII.295) all gave IC_{50} values of 0.02–0.06 μM as compared with 0.07 μM for the parent amine (VIII.275). A similar range of IC_{50} values was obtained for the N^{10}-formyl derivatives (VIII.284)–(VIII.289), showing that DHFR binding is minimally affected by this modification, despite the fact that conversion of N^{10} to an amide would clearly be expected to alter the basicity of this nitrogen. However, despite the similarities of their IC_{50} values against DHFR, these compounds exhibited profound differences in their ability to inhibit cell growth. While the N^{δ}-acetyl ((VIII.290), IC_{50} = 0.017 μM) and N^{δ}-hemisuccinoyl ((VIII.291), IC_{50} = 0.037 μM) derivatives were about 10-fold more potent than the parent amine (IC_{50} = 1.3 μM), the N^{δ}-benzoyl ((VIII.292), IC_{50} = 0.00089 μM) and N^{δ}-hemiphthaloyl ((VIII.295), IC_{50} = 0.00075 μM) derivatives were much more potent, and, in fact, even exceeded the potency of MTX and AMT. Interestingly, the N^{δ}-(4-chlorobenzoyl) derivative (VIII.293) (IC_{50} = 0.0032 μM) was several-times less potent than (VIII.292), while the potency of the N^{δ}-(3,4-dichlorobenzoyl) derivative (VIII.294) (IC_{50} = 0.032 μM) fell to the level of the non-aromatic amides. Substitution of the aromatic ring with lipophilic (and electron withdrawing) halogen substituents therefore seemed to be unfavourable. Each of the N^{10}-formyl derivatives tested proved less active than its N^{10}-unsubstituted counterpart, suggesting that N^{10}-formylation probably has a greater effect on cellular uptake than on binding to DHFR. Several of the acylated derivatives of (VIII.275) were also tested against CEM human leukaemic lymphoblasts, and once again the N^{δ}-benzoyl and N^{δ}-hemiphthaloyl derivatives (VIII.292) and (VIII.295) proved to be excellent inhibitors, with IC_{50} values of 0.0066 and 0.0043 μM, respectively, as compared with 0.022 μM for MTX. Finally, in assays [369] against two human head and neck squamous cell carcinoma lines (SCC15, SCC25) [262], (VIII.295) was found to have an IC_{50} value of 0.001 μM, while the potency of MTX was 3-fold lower against the SCC15 cells and 10-fold lower against the SCC25 cells.

While none of the acylated derivatives of (VIII.275) succeeded in overcoming MTX resistance in the highly resistant L1210/R81 cell line, the aromatic amides (VIII.292) and (VIII.295) were remarkably effective [369] in preventing the growth of the CEM/MTX cell line, which is *ca.* 200-fold resistant by virtue of defects in transport and polyglutamylation [263, 304, 372], and the growth of the squamous cell carcinoma sublines SCC15/R1 and SCC25/R1, which have been shown to be even more heterogeneous than the CEM/MTX cells in their resistance phenotype [262]. The IC_{50} values of (VIII.292) and (VIII.295) against CEM cells were 1.1 and 0.66 μM, respectively, as compared with 6.6 μM for MTX. More strikingly, the IC_{50} value of (VIII.295) against SCC15/R1 cells was 0.0040 μM as compared with 0.58 μM for MTX, while the IC_{50} values of (VIII.295) and MTX against the SCC25/R1 cells were 0.0013 and 0.15 μM, respectively. An unprecedented feature of the latter results was that the activity of (VIII.295) against MTX-resistant SCC cells *exceeded that of MTX against the parental cells.* The biochemical basis for this activity against MTX-resistant cells has not yet been elucidated, though it has been proposed that intracellular cleavage of the N^{δ}-hemiphthaloyl group would generate (VIII.275), which could then act as a 'self potentiating antifolate'. According to this model (which remains at the moment only a hypothesis), any free (VIII.275) in the cell in excess of the amount bound to DHFR might interfere with the polyglutamylation of cellular reduced folates and thus diminish the ability of these species to participate in *de novo* synthesis of the nucleotide precursors of DNA.

The N^{ω}-haloacetyl derivatives (VIII.276)–(VIII.280) have also been tested as DHFR inhibitors and cell growth inhibitors [365, 369], but have been of limited interest except for the fact that the more chemically reactive members of the series, the iodoacetamide (VIII.276) and the bromoacetamides (VIII.277) and (VIII.280), can bind covalently to DHFR, probably at the active site. Covalent bond formation appears to be relatively slow, however, and there is likely to be considerable loss of alkylating activity due to solvolysis of the haloacetyl group. In a radioligand binding assay using non-purified DHFR from L1210 cells and purified DHFR from *L. casei*, the IC_{50} values of (VIII.276) were found to be 9.8 and 31 nM, respectively, as compared with IC_{50} values of 11 and 122 nM for the parent amine (VIII.264) and 2.7 and 15 nM for MTX [365]. Since the assays was performed over a short time (< 15 min), the IC_{50} values observed for (VIII.276) presumably represented only *reversible* inhibition. To determine whether (VIII.276) bound covalently to DHFR, *L. casei* enzyme was incubated for 6 h in the presence of 1.0 μM drug at pH 7.4, and residual DHFR activity was measured at various intermediate time points by competitive [^{3}H]MTX-binding assay. After 5.5 h, the

ability of the enzyme to bind [^{3}H]MTX had decreased to 4% of control values. Loss of binding was linear, and was insignificant when the ligand was the N^{ϵ}-Cbz rather than N^{ϵ}-iodoacetyl derivative. Loss of binding was likewise not observed with iodoacetamide at the same molar ratio. When the half-life for [^{3}H]MTX binding was plotted as a function of pH, a sigmoid curve was obtained, with an inflection point estimated to lie at pH 7.2, which is close to the pK_a for an imidazole nitrogen in histidine. It was suggested that (VIII.276) binds rapidly to the enzyme in a reversible manner, and that this is followed by slower formation of a covalent bond to a histidine residue near the active site, possibly after a conformational change in the enzyme to allow this reaction to occur. It was postulated that the amino-acid residue undergoing alkylation in the *L. casei* enzyme was His-28, and this was subsequently confirmed by Freisheim *et al.* [381], who hydrolyzed the covalently modified enzyme and found *N*-carboxymethyl-L-histidine (both possible isomers) to be present in the fragment in which His-28 normally resides. In a later study [382], (VIII.276) was reported to also bind covalently to DHFR from chicken liver and human lymphoblasts (WI-L2 cells), apparently by alkylation of cysteine. This was surprising, since the single cysteine residue in these enzymes is thought to be outside the active site, where it cannot be easily reached without a major change in protein secondary structure.

In the most recent work on the haloacetyl derivatives [369], it was reported that the bromoacetamides (VIII.277) and (VIII.279) appeared to bind covalently to DHFR from L1210 cells, as well as to the enzyme from *Candida albicans*. In the case of the L1210 enzyme, the reaction mixture, with or without added NADPH, was incubated for 19 h, after which the enzyme was denatured with urea to release noncovalently bound ligands, purified on a gel-filtration column, and finally examined by quantitative ultraviolet spectrophotometry. From a comparison of the absorption coefficients at 302 nm for control and treated enzyme, the extent of covalent modification was estimated to be 20 mol% for the lysine derivative (VIII.277) and 60 mol% for the ornithine derivative (VIII.279). However, when the covalently modified enzyme was hydrolyzed with acid and quantitative amino-acid analysis was performed, no carboxymethylated species could be detected (including especially *S*-carboxymethyl-L-cysteine). To explain this, it was proposed that alkylation of the L1210 enzyme had produced an *acid-labile* modification, possibly at Glu-62. In the experiments using *Candida* enzyme, incubation with excess drug in the absence of NADPH, followed by passage through a gel-filtration column (to remove noncovalently bound ligand) and addition of the recovered protein to dihydrofolate and NADPH, was found to cause significant loss of functional activity. The ornithine derivative (VIII.279) produced

a 75% loss of activity after 6 h of incubation, whereas with the lysine derivative (VIII.277) this decrease was 60% after 4 h. From an analysis of steady-state inhibition velocities in the reduction of dihydrofolate in the presence of (VIII.279) and NADPH, the K_i for reversible inhibition of the *Candida* enzyme was estimated to be 0.32 nM as compared with 0.27 nM for MTX. The chloroacetamides (VIII.278) and (VIII.280) gave no evidence of covalent binding to either the L1210 enzyme or *Candida* enzyme.

Biochemical and biological data on derivatives of (VIII.264) and (VIII.268) with fluorescent ligands and potential photoaffinity labels at the ω-position have likewise been reported. The fluoresceinated derivative (VIII.306) (PT430) was reported to inhibit DHFR from *L. casei* with an IC_{50} value of 200–220 μM and enzyme from L1210 cells with an IC_{50} value of 15–25 μM [326]. The corresponding values for MTX were 15–20 and 7–9 μM, respectively. Thus, replacement of the γ-carboxyl group in MTX by a bulky *N*-(4′-fluoresceinyl)thioureidomethyl group markedly decreased binding to this bacterial enzyme, but had a relatively minor effect on binding to the mammalian enzyme. The fluorescence of PT430 was characteristically enhanced on binding to DHFR, and it was possible to calculate a K_D value from the fluorescence enhancement at different ligand concentrations. The K_D for binding to *L. casei* enzyme varied from 4.7 nM at pH 7.4 to 1.4 nM at pH 6.0. In assays of L1210 cell growth inhibition, PT430 had an IC_{50} value of 3 μM as compared with 0.03 μM for MTX. Thus, there was a 100-fold loss of growth-inhibitory potency as compared with the less than 3-fold decrease in binding to DHFR. As with so many other compounds for which this type of discrepancy is observed, the decreased ability of PT430 to inhibit cell growth in comparison with MTX was ascribed to differences in uptake. Kinetic data for the transport of PT430 in CEM cells suggest that this compound is taken up partly by the same carrier-dependent pathway as MTX, but also via another mechanism such as diffusion. The ability of P430 to serve as a reporter for DHFR has been used to differentiate DHFR-overproducing MTX-resistant cells from wild-type MTX-sensitive cells by flow cytometry [326]. The ability of this compound to also serve as a probe of MTX active transport has likewise been used in flow cytometric studies of MTX-resistant cells with a transport defect [372].

The binding of the dansyl derivative (VIII.307) to DHFR from *L. casei*, human lymphoblasts (WI-L2 cells), and chicken liver has been studied [367, 373, 374]. The ratio [I]/[E] for extrapolated 100% inhibition of enzyme activity by spectrophotometric measurement was found to be 1.0–1.4 for (VIII.307), 1.9–3.5 for (VIII.264), and 1.0 for MTX. This demonstrated that the dansyl group was well tolerated by the active site of DHFR, and that the

dansylated ornithine analogue had a somewhat higher affinity than the ornithine analogue itself. In subsequent work [375] the IC_{50} of (VIII.307) against L1210 enzyme was determined to be 98 nM, while that of MTX was 40 nM. As with PT430 (*vide supra*), the fluorescence intensity of (VIII.307) was enhanced on binding to the enzyme. Moreover, binding elicited a blue shift in the emission wavelength, suggesting that the fluorophore lies in a hydrophobic environment in the enzyme active site. Transport experiments were also performed with (VIII.307) and MTX. The K_t for MTX transport in L1210 cells was determined to be 7.3 μM, while the K_i of (VIII.307) for inhibition of MTX transport was 7.0 μM. Similar values were obtained against L1210/R71 cells, a resistant subline with elevated DHFR activity. It thus appeared that (VIII.307) had an affinity comparable to that of MTX for the transport system. In growth inhibition assays against L1210 cells, (VIII.307) was found to have an IC_{50} value of 0.36 μM as compared with 0.029 μM for MTX, while in assays against MTX-resistant L210/R71 cells the corresponding IC_{50} values were 340 and 37 μM. Since MTX and (VIII.307) displayed a comparable 10- to 15-fold difference against the two cell lines, it was clear that (VIII.307) did not have the capacity to overcome MTX resistance.

The ability of the N^{ω}-azidosalicylyl derivatives (VIII.309)–(VIII.312) to inhibit DHFR has been compared to that of MTX and the parent amines (VIII.264) and (VIII.268) [377]. The following IC_{50} values were obtained (data against *L. casei* and L1210, respectively): (VIII.264), 54 and 21 nM; (VIII.309), 68 and 29 nM; (VIII.311), 85 and 44 nM; (VIII.268), 62 and 50 nM; (VIII.310), 58 and 40 nM; (VIII.312), 50 and 34 nM; MTX, 25 nM. Thus, in a striking illustration of the subtle structure-activity effects that can exist among side-chain analogues of MTX, N^{ε}-salicylation of the lysine derivative decreased enzyme binding, while N^{δ}-salicylation of the ornithine analogue had the opposite effect. Irradation of binary enzyme-inhibitor complexes for 20 min at 4 °C with long-wavelength ultraviolet light led to covalent modification of the enzyme with an efficiency of approximately 35%. The identity of the covalently modified residues was not determined in this initial study, and it was pointed out that nitrenes generated on photocatalytic decomposition of azides are typically very reactive and tend to yield adducts by insertion into a variety of bonds. In a subsequent modification aimed at increasing the specificity of labeling, the complex between ^{125}I-(VIII.311) and *L. casei* DHFR was irradiated [377] for as little as 1 min, with a decrease in lebelling efficiency to 8%. Denaturation of the enzyme followed by cyanogen bromide cleavage yielded mainly one radioactive photolabelled peptide. From an analysis of the computer-generated three-dimensional structure of chicken liver enzyme, it was possible to suggest that the region most likely to be modified was around residues 63–65 (Lys-Asn-Arg).

Elegant photolabelling studies with ^{125}I-(VIII.311) were also performed by Price and Freisheim [379] using intact L1210 cells. Cellular accumulation of the radioiodinated compound over a period of 12–20 h was similar to MTX; in fact, the transport K_t for (VIII.311) was 5.5-fold lower than that of MTX. Uptake was temperature-dependent, and was inhibited by *p*-(chloromercuri)benzenesulphonate (pCMS). Uptake of 0.2 μM (VIII.311) was inhibited by 90% in the presence of 50 μM MTX and by 50% in the presence of 1.0 μM MTX. When the cells were incubated with (VIII.311) at 4 °C, the drug was prevented from entering the cells but could be made to react covalently with a membrane component by irradiation for 30 s with long-wavelength ultraviolet light. The covalently modified plasma membrane was then solubilized with detergent and subjected to polyacrylamide gel electrophoresis and radioautography. A membrane protein with a molecular mass of 46–48 kDa was identified as the likely carrier protein for MTX, since its photolabelling was abolished by a 1000-fold molar excess of MTX. Binding of (VIII.311) to the 46–48 kDa membrane protein was also inhibited by 5-methyltetrahydrofolate. When irradiation was conducted at 37 °C instead of 4 °C, the photoaffinity probe did not bind to the membrane, but instead reacted with two soluble cytosolic proteins of 38 and 21 kDa. Additional support for the conclusion that (VIII.311) was a specific probe for the MTX carrier protein came from the finding that MTX-resistant L1210/R81 cells, which are virtually incapable of actively transporting MTX [49], did not form the 46–48 kDa adduct.

CONCLUSION

> "It is the purpose of this paper to record the results of clinical and hematologic studies on 5 children with acute leukaemia treated by the intramuscular injection of a synthetic compound, 4-aminopteroylglutamic acid (aminopterin). This substance is an antagonist of folic acid regarding growth of *Streptococcus faecalis* R."

> "Clinical, hematologic and histologic details on 5 patients with acute leukaemia treated with aminopterin, selected from a group of 16 patients so treated, form the basis of this paper. It is again emphasized that these remissions are temporary in character, and that the substance is toxic and may be productive of even greater disturbances than has been encountered so far in our studies. No evidence has been mentioned in this report that would justify the suggestion of the term 'cure' of acute leukaemia in children. A promising direction for further research concerning the nature and treatment of acute leukaemia in children appears to have been established by the observations reported."

Forty years have elapsed since these cautiously prophetic words were used by Dr. Sidney Farber and his colleagues [1] to report in a short clinical paper in the New England Journal of Medicine that several children with acute leukaemia who were given a new experimental drug called aminopterin had undergone temporary clinical remission, with many of the characteristic signs of haematologic disease disappearing almost completely. It is difficult for people today to appreciate the enormous excitement this brief paper aroused in the scientific and lay community.

Though aminopterin was the drug actually used in this clinical study, it was soon supplanted by methotrexate, for a number of practical reasons whose history need not be recounted here. Suffice it to say that, rightly or wrongly, methotrexate immediately became the 'gold standard' by which all future folic acid antagonists would be judged. Beyond that, however, the greater historical importance of methotrexate lay in the fact that it launched a new era in cancer treatment, in which it was envisaged that not only children but also adults with cancer could be treated with a whole arsenal of antimetabolites designed to selectively destroy malignant cells while inflicting minimal, or at least tolerable, injury to normal host tissues. With the advent of other antimetabolites such as 6-mercaptopurine and 5-fluoropyrimidine, and of other classes of drugs whose action was more directly targeted against DNA, such as the alkylating agents and intercalating agents, chemotherapy became a powerful member of the triad of weapons that could be deployed by medical scientists in their battle against cancer.

In reading the carefully worded paragraph with which Farber and his colleagues concluded their paper, it is difficult to know how much of the future history of research and development in the antifolate area they were able, even then, to anticipate. The theoretical framework for the use of antimetabolites was already in place in 1948, and it is likely that at least some of the basic scientists working in the field, such Dr. Y. SubbaRow at Lederle Laboratories and Dr. George Hitchings at Burroughs-Wellcome, were already cognizant of the possibility of acquired resistance. Implied anticipation of the problem at the clinical level was evident in the statement that *"...these remissions are temporary in character."* The major problem of selectivity of action was also openly addressed in the statement that *"...the substance is toxic."* The remarkably foresighted statement that *"the substance...may be productive of even greater disturbances than have been encountered thus far in our studies"* then followed. What was being specifically meant here is somewhat unclear, but in retrospect, one can certainly view this interesting phraseology as an expression of a concern that the drug had the potential to alter the histopathology of a malignancy in a manner that could, in the end, make it even more

destructive. Most important of all, however, in terms of the *medicinal chemistry* of antifolates over the next four decades, was the final sentence; *"A promising direction for future research..."* Here there can be little doubt as to the meaning: aminopterin was to be only the opening shot in a long campaign, and was not to be viewed as a definitive weapon. Other agents had to be sought–agents with greater therapeutic power, a broader spectrum of activity, and fewer side-effects.

Methotrexate quickly garnered widespread clinical acclaim as a substitute for aminopterin, and eventually came to be used even against certain diseases other than cancer such as psoriasis and rheumatoid arthritis. Remarkably enough, however, despite years of painstaking research, there remain to this day a number of fundamental questions about the precise mechanism of action of methotrexate and the basis of its lethality to cells. Whether or not Dr. Farber and his associates imagined how many antifolate compounds would ultimately be synthesized and tested in attempts to answer these questions will never be known. As this review demonstrates, the number of new compounds studied in this connection has indeed been immense. More important than the mere number of compounds, however, is the remarkable degree to which rationales underlying the design of these compounds have grown in elegance and sophistication over the years. Indeed, it seems fair to say that some of the most important insights gained regarding the mode of action of methotrexate have actually come about as a result of studies on methotrexate *analogues*.

It is undoubtedly true that some of the analogues described in this review were made originally on the basis of concepts that may be considered today to be naive, e.g., the interchanging of carbon and nitrogen atoms in the pteridine moiety or the bridge, or the replacement of the glutamate side-chain by other amino acids. With the unfolding of new knowledge about the biochemistry of folates, however, and especially with the ability to use powerful tools of modern molecular biology in pharmaceutical research, there is good reason to hope that, in the years ahead, exciting new members of the antifolate family will join methotrexate as 'commonly used drugs' for the treatment of cancer and other human diseases.

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2 Recent Developments in Aromatase Inhibition as a Potential Treatment for Oestrogen-Dependent Breast Cancer

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INTRODUCTION

Breast cancer is one of the most common malignancies in women [1]. Statistics in the U.S.A. alone show that over 100,000 new cases are diagnosed and 40,000 deaths occur each year [2]. The incidence increases with age, 75% of cases occurring between the ages 40 and 75. Eight out of ten women with the disease will die from it [3]. Although 90% of patients present with a cancer that is clinically localized to the breast and the axillary lymph nodes, the majority who eventually die of the disease suffer from systemic metastases in spite of local control [4].

The influence of ovarian hormones, especially oesterogens, on the development of breast cancer has long been recognized. In laboratory animals, oestrogen administration is an effective means of inducing mammary tumours, while in human patients the introduction of artificial menopause for reasons other than breast cancer can reduce the subsequent incidence of breast cancer by up to 75% [5].

Oestrogens interact with the specific receptors to form an oestrogen-receptor complex which migrates to the nucleus and interacts with the chromatin [6] (a more recent view suggests that the receptor occurs in the nucleus [7]). This results in the synthesis of RNA and subsequently of proteins which exert several effects, amongst which is the promotion of cell division [8].

Although the growth of breast cancer may be accelerated by increased endogenous oestrogens, and may regress when endogenous oestrogen is reduced or antagonized, it has not been established that the cause of the development of breast cancer is due to the increased levels of endogenous oestrogenic stimulation [9]. However, numerous attempts to involve one or other, or combinations of the oestrogens, oestradiol (E2), oestrone (E1) and oestriol (E3) as the provocative agent have been made, for example, the 'oestriol hypothesis' [10, 11]. This theory suggested that the risk of developing breast cancer is inversely proportional to the concentration of E3 relative to

the total E2 and E1 produced by a woman in the decade following menarche. The protective effect of progesterone led to the 'oestrogen-window hypothesis' [9, 12, 13], where it is suggested that the breast cancer is more easily induced when levels of progesterone are low relative to the levels of oestrogen, that is, the window is open. However, during pregnancy, progesterone levels are high and tumour induction is less likely, that is, the window is closed. Although there is some support for these and other related theories, none of them has been fully established, so leaving unresolved the role of oestrogens and the relevance of oestrogen ratios and the progesterone levels on cancer induction.

There are world-wide differences in the distribution of incidence of breast cancer and some of these may be related to lifestyle, diet and obesity [14, 15].

One of the factors accounting for geographical differences in the occurrence of the disease seems to be related to the average fat intake [14, 16]. In one study an association has been established between fat intake and breast cancer risk in normal women and breast cancer patients [17]. The cholesterol content of the fat does not seem to be an important factor [18]. The mechanism whereby fat may increase the risk of breast cancer is not clear and suggestions range from effects on serum hormone-binding globulin levels [19] or levels of 16-hydroxytestosterone [20] (a possible carcinogen) or an increase in oestrogen levels resulting from steroid metabolism by gut bacteria [21].

Women who have not had a child before their thirtieth birthday are one-and-a-half times more likely to develop breast cancer than women who have [22]. Having additional children, particularly if at an early age, may be protective against breast cancer [23]. Recent investigations [24] into the first pregnancies and long-term hormonal changes suggest that the protective mechanism for breast cancer may be related to the duration of the hormonal change rather than the change itself.

Other factors affecting the incidence of breast cancer are family history [25, 26], early menarche and/or late menopause [27, 28], receiving long-term oestrogen replacement therapy [28], obesity [22] and an increase in the number of ovulatory cycles [29].

The supply of oestrogen produced endogenously for the continued maintenance of tumour growth may be supplemented by the cancerous tissue itself (see review by Miller [30]). Over half [31] of human breast cancer cells synthesize oestrogen from androgen precursors *in vitro* [32–35]. Studies [35] on the synthesis *in situ* of oestrogens from human breast tumours by either the aromatase enzyme (from androstenedione) or a sulphatase enzyme (from oestrone sulphate) have shown that the latter pathway predominates. The sulphatase:aromatase activity ratio is about 10 at normal *in vivo* concen-

trations of substrates. Attempts to quantify the amount of oestradiol produced locally in relation to that concentrated in the tumour by uptake of circulating plasma oestradiol have not been described.

The actual biological significance of aromatase activity in breast cancer tumours is not clear. Studies have shown that there is no correlation between aromatase activity and oestrogen receptor concentrations in breast tumours [36] or the size of primary tumours [37].

THERAPY

Surgical therapy

Following detection of the disease, the progression is determined by the TNM system (tumour, nodes, metastases) and the most appropriate therapy for the individual concerned is selected.

Surgery is the normal method for local control of the breast cancer and, if used at an early stage, produces a cure in many patients. Treatment may involve removal of the primary tumour, radical mastectomy with radiation, or tylectomy. However, after surgery, more than half the patients will relapse, with a mean duration of survival of about 3 years [38]. The selection of appropriate follow-up treatment for the patient who develops recurrence or metastases will have a considerable influence on the patient's duration of survival. A treatment scheme for patients with metastatic breast cancer has been summarized by Allegra [39] and a summary of factors influencing selection of therapy by Clarysse [40].

Endocrine ablation may be considered a suitable means of treatment. Oophorectomy has been found effective in premenopausal women [39]; adrenalectomy and hypophysectomy have been used [41] in oophorectomised women to give objective tumour regression in 50–60% of women classified as oestrogen-receptor-positive [42] (see later). However, even after these ablative treatments, patients may still continue to produce oestrogens in significant amounts [43]. Consequently, medicinal therapy is almost always considered, to stem further growth and attempt to curtail metastases of the disease.

Medicinal therapy

The type of therapy suitable is usually guided by the extent to which the oestrogen receptors are present in the cancerous breast tissue [44]. A patient with a high concentration of receptor protein (that is, more than 5 fmol

receptor protein/mg breast tissue) is designated as being oestrogen-receptor-positive (ER +) while one below this value is regarded as receptor-negative (ER −).

Approximately two-thirds of primary breast cancers are ER + [45]. Oestrogen receptor status is important, as it determines whether or not the patient can benefit from hormonal therapy and also provides information on the history of the cancer, since ER + patients show a longer disease-free interval and total survival than ER − patients. About 60% of ER + patients and 10% of ER − patients may respond to hormonal therapy [45, 46]. Patients with ER − tumours have very little chance of remission with endocrine therapy; ablative procedures are avoided and replaced by cytotoxic therapy [47].

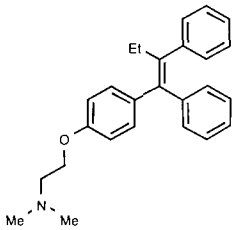
The presence of progesterone receptors in cancerous tissue improves the diagnosis of the disease, as there is an inverse relationship between the concentration of progesterone receptor material and the frequency of the metastasis [48]. Patients with oestrogen and progesterone receptors have about 75–80% chance of responding to hormonal therapy [49].

Anti-oestrogens

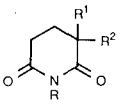
Oestrogens are implicated in both the induction and maintenance of breast cancer and inhibition of oestrogen activity or biosynthesis has gained support at the expense of earlier endocrine therapy with high dose oestrogens, androgens, corticosteroids or progestins and their often severe side-effects. The medicinal endocrine therapy of choice in the treatment of post-menopausal women with breast cancer, irrespective of receptor status, is tamoxifen (1). This drug competes with oestradiol for the ER receptor, thus inhibiting the formation of the ER complex which, as described previously, governs tumour tissue synthesis. Tamoxifen may additionally act by reducing new receptor synthesis [50]. Overall response rates for tamoxifen are reported [51] to vary from 16 to 50% with a mean of 32%. Patients eventually become refractory to the drug over a period of 6–17 months [51, 52]. 'Second line' drugs used in the treatment of tamoxifen-resistant tumours are aromatase inhibitors, which lower endogenous oestrogen levels blocking the conversion of androstenedione and testosterone to oestrone and oestradiol, respectively.

Non-steroidal reversible inhibitors of aromatase

Initial attempts to develop aromatase inhibitors for use in the treatment of oestrogen-dependent breast cancer involved the use of synthetic steroid analogues of the natural substrates androstenedione and testosterone [53]. Such steroidal inhibitors are reviewed in on p. 272. An alternative approach to the design of aromatase inhibitors was suggested by the discovery [54–56]

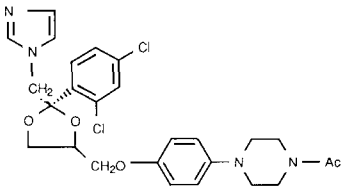


(1)



(2) R= H R^1= 4'-aminophenyl R^2= Et

(3) R= H R^1= 4'-pyridyl R^2= Et



(4)

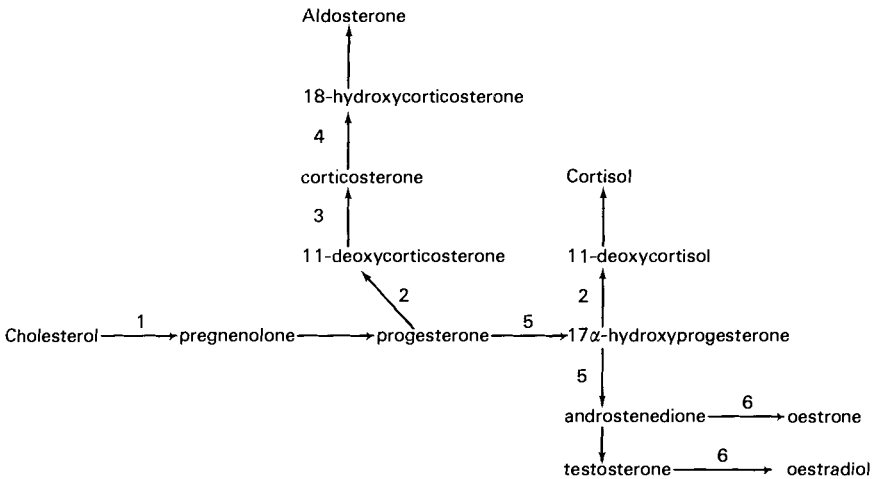
that non-steroidal drugs such as aminoglutethimide (AG, Orimeten®, (2)) and ketoconazole (Nizoral® (4)) are inhibitors of this enzyme. Although these drugs are relatively non-specific, such proven inhibitors could provide a basis for the development of safe and more selective inhibitors. Three major groups of non-steroidal inhibitors may be identified: aniline derivatives such as AG and related compounds; imidazole- or triazole-containing drugs such as ketoconazole; and synthetic pyrimidine derivatives.

Pharmacology of aminoglutethimide and related compounds

AG binds to cytochrome *P*-450 to produce a Type II optical difference spectrum with an absorption peak occurring between 425 and 435 nm and a trough at 390 nm [55, 57, 58]. Such spectra are characteristic of nitrogen-containing compounds such as aniline, and probably reflect electron transfer between the haem iron and the nitrogen atom of the exogenous compound [59, 60]. This binding to the haem group could result in an interaction between AG and the substrate binding site on the *P*-450 enzyme, thus producing competitive inhibition [55]. This is supported by the observation that in microsomes prepared from bovine corpus luteum [57] and human placenta [55], the enzyme-inhibiting activity of the D- and L-enantiomers of AG correlate well with the spectral affinity constants; in both cases the D-isomer is a more potent inhibitor than the L-isomer.

The low specificity of AG for the cytochrome *P*-450 dependent enzymes results in a broad spectrum of enzyme inhibition [61]. Several key enzymes in the steroidogenic pathways producing oestrogens from cholesterol are inhibited (*Figure 2.1*). As well as aromatase, AG inhibits the cholesterol side-chain cleavage enzyme ($C_{20,22}$ lyase; CSCC) which converts cholesterol into pregnenolone [62], the 11-hydroxylation of 11-deoxycortisol [63], 21-hydroxylation of 17α-hydroxyprogesterone [64], and the 18-hydroxylation of corticosterone [64, 65]. *In vitro* AG is most effective against 18-hydroxylase, with effective concentrations being an order of magnitude lower than those required to inhibit CSCC, and higher concentrations still being needed to inhibit 11-hydroxylase [61]. In addition, AG inhibits the synthesis of the thyroid hormones triiodothyronine and thyroxine *in vitro*, and produces clinical signs of hypothyroidism *in vivo* [66]. It also induces the metabolism of many steroids and drugs *in vivo* [67] and, paradoxically, it has been reported to induce aromatase activity in breast tumour tissue *in vitro* [68].

The lack of specificity of AG for aromatase presents problems in its clinical



1. Cholesterol side-chain cleavage (CSCC), $C_{20,22}$-lyase
2. 21β-hydroxylase
3. 11β-hydroxylase
4. 18β-hydroxylase
5. 17β-hydroxylase/$C_{17,20}$-lyase
6. Aromatase complex

Figure 2.1. Key P*-450-dependent enzymes involved in the biosynthesis of steroids from cholesterol.*

use (see below). Due to its inhibitory action on CSCC, corticosteroid production is reduced in patients treated with AG and therefore concomitant cortisol (hydrocortisone) replacement is necessary [61]. For this reason, attempts have been made to develop analogues with less activity against CSCC [69–74]. Reliable *in vitro* assays for both aromatase and CSCC have been described which have been invaluable in the development of such compounds. The aromatase assay most commonly involves the measurement of tritiated water release from [1,2-^{3}H]androstenedione or testosterone; the source of the enzyme is usually human placenta [55] or ovaries from rats treated with pregnant mare serum gonadotrophin (PMSG) [75]. The CSCC assay measures the production of [^{14}C]isocaproic acid from labelled cholesterol by microsomes prepared from bovine adrenal glands [76]. Using these assays much information has been obtained on the structural requirements for a potent selective inhibitor of aromatase.

Aromatase inhibition by compounds such as AG appears to be dependent on the presence of an amino or other basic group [69, 70] and especially when this group is in the 4′-position of the phenyl ring. Relocation of the amino group elsewhere in the phenyl ring produces compounds with less inhibitory activity towards aromatase but enhanced activity towards CSCC [70]. Thus, in the *in vitro* CSCC assay AG has a K_i of 1400 nM, while the 3-(3-aminophenyl)-3-ethylpiperidine-2,6-dione and 1-amino-3-ethyl-(3-aminophenyl)-piperidine-2,6-dione isomers have K_i values of 1300 nM and 4600 nM, respectively. The latter compound is ineffective against aromatase, while the *meta*-AG analogue is a very weak inhibitor (IC_{50} value of approximately 30 μg/ml, cf. *ca.* 2 μg/ml for AG [70]). Metabolites of AG in which the amino group is modified show reduced or no inhibitory activity [69]. Replacement of the 3-*p*-phenyl substituent with a 3-pyridyl group in AG produces compounds which are stronger bases than AG and might therefore be expected to be more potent aromatase inhibitors [71]. The 2′- and 3′-pyridyl analogues are ineffective against aromatase, while the 4′-pyridyl analogue (3-ethyl-3-(4-pyridyl)piperidine-2,6-dione, pyridoglutethimide, PG, (3)) is a potent inhibitor, although less so than AG (K_i for AG and PG are 600 nM and 1100 nM respectively; K_m for testosterone, 130 nM). Furthermore, this analogue does not inhibit CSCC at concentrations of up to 50 μg/ml [71].

Pyridoglutethimide therefore seems a potentially useful aromatase inhibitor *in vitro*. *In vivo*, it reduces tumour growth in rats treated with nitrosomethylurea [77]. Preliminary studies have been made of its metabolism and pharmacokinetics in rats and rabbits [78]. It has a half-life of 6 h in rats and 16.4 h in rabbits, and in both species the sole metabolite is the *N*-oxide. Limited data from a human subject who received a single oral dose of 50 mg suggest that

the same pathway is responsible for PG elimination in man [78]. Behavioural tests in mice have shown that PG has few side-effects on the central nervous system at doses of up to 300 mg/kg, while smaller doses (100–200 mg/kg) of AG produce severe lethargy or ataxia [77]. Since such side-effects are a major limiting factor in the clinical use of AG, this could have useful implications in the therapeutic use of PG.

Since PG is a slightly weaker aromatase inhibitor *in vitro* than AG, attempts have been made to produce more potent analogues. Two series of compounds have been described in which alkyl groups are substituted either on the piperidine nitrogen atom (1-alkyl- or *N*-alkyl derivatives) or the 3-position [79, 80].

These compounds retain the selectivity of PG for aromatase, and show increased inhibitory potency as the length of the alkyl group is increased. In both the 1- and 3-substituted series the optimum length of the alkyl substituent is 8 carbon atoms; 1-octylpyridoglutethimide has a K_i of 120 nM when testosterone is used as a substrate (cf. 1100 nM for PG) and 480 nM when androstenedione is used as the substrate [80]. For 3-octylpyridoglutethimide the corresponding figures are 90 nM and 200 nM, respectively. *In vivo*, both compounds have shorter half-lives (< 1 h) in rats and rabbits than pyridoglutethimide, and undergo more complex metabolism [81]. If the same is true in humans, this could limit the potential usefulness of these compounds. To date, there is no information available concerning the effects of these compounds on plasma oestrogen concentrations in animals or humans, or on aromatase activity, *in vivo*.

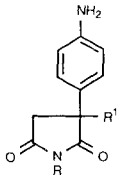
A series of 1-alkyl analogues of AG has been described by the same workers [74]. As with the pyridoglutethimide analogues, optimal aromatase inhibition is produced by the hexyl and octyl analogues; the *n*-propyl analogue, however, has the lowest activity against CSCC and the greatest specificity for aromatase (IC_{50} values of 500 nM for aromatase and 22,000 nM for CSCC).

An extensive range of analogues of AG has been described by Hartmann and Batzl [72, 73]. In one series, the 3-ethyl group of AG was replaced by another alkyl group to produce a series of compounds analogous to the C-alkyl derivatives of PG described above [72]. Increasing the length of the alkyl group from 2 to 7 carbon atoms tended to increase the inhibition of human placental aromatase *in vitro*, with the 3-isopentyl analogue being the most potent. This compound was about 93-fold more potent (in terms of IC_{50} values) than AG [72]. Moreover, most of these compounds showed either a similar inhibition of bovine adrenal CSCC to AG or a reduced inhibition. Thus, when tested at a single inhibitor concentration of 25 μM, AG showed 57% inhibition of CSCC while the analogues with chain lengths of between

5 and 7 carbon atoms showed approximately 20–30% inhibition. The analogues with the greatest potency *in vitro* have been further tested in animal studies. In PMSG (pregnant mare serum gonadotrophin)-treated rats, compounds with alkyl groups of up to 7 carbon atoms reduced ovarian vein oestradiol concentrations by between 79 and 94% after a single administration; AG reduced concentrations by only 67%. The 3-*n*-hexyl and 3-*n*-heptyl analogues, however, were roughly equipotent with AG *in vivo*, despite being up to 25-fold more active *in vitro*. Some of the compounds with the greatest activity in the PMSG-treated rat system were also tested for their ability to reduce testosterone-stimulated tumour growth in ovariectomized rats bearing tumours induced by 7,12-dimethylbenz[*a*]anthracene (sometimes erroneously called 9,10-dimethyl-1,2-benzanthracene, DMBA) [72]. All of the compounds tested inhibited testosterone-stimulated tumour growth more strongly than AG.

Batzl and Hartmann have also prepared a series of analogues of AG in which the alkyl group is substituted at the 4-position [73]. Compounds bearing a 4-methyl, -ethyl or -propyl group have only 10–30% of the inhibitory activity of AG *in vitro*. *In vivo*, however, the 4-ethyl analogue reduces plasma oestradiol concentrations in PMSG-treated rats to a similar extent to AG.

The glutarimide ring of AG is not essential for aromatase inhibition [69], since 4-cyclohexylaniline, in which this ring is replaced by a cyclohexyl moiety, is an effective inhibitor of aromatase *in vitro* [82] with a K_i of 140 nM against androstenedione (cf. 300 nM for AG). Since there is evidence that some of the side-effects of AG, such as ataxia and skin rashes, are attributable to the glutarimide ring [83], alteration could produce analogues which inhibit aromatase but are devoid of such side-effects. A series of such compounds has been described [69, 84] in which the piperidine-2,6-dione ring is replaced by a pyrrolidine-2,5-dione (succinimide) structure. Two of these, 3-(4-aminophenyl)pyrrolidine-2,5-dione (5) and 3-(4-aminophenyl)-3-ethylpyrrolidine-2,5-dione (6), proved to be effective inhibitors of aromatase *in vitro*, albeit less so than AG; the K_i values (testosterone as substrate) were 680 nM for AG, 950 nM for the parent compound, and 1,000 nM for the 3-ethyl analogue [84]. A third compound, 3-(4-aminophenyl)-3-ethyl-1-methylpyrrolidine-2,5-dione (7), was only a weak inhibitor of aromatase *in vitro* [84], but apparently undergoes *N*-demethylation *in vivo* to produce the 3-ethyl analogue [85]. *In vitro*, the parent compound did not inhibit CSCC, while the 3-ethyl analogue inhibited the enzyme by 38% at a concentration of 50 μg/ml, whereas AG at the same concentration caused 83% inhibition [84]. The 3-ethyl analogue has been tested in PMSG-treated rats [85, 86], and has been found to reduce plasma oestradiol concentrations by up to 97%. Aromatase



(5) R= H R^1= H

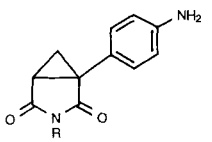
(6) R= H R^1= Et

(7) R= Me R^1= Et

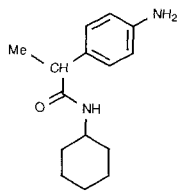
activity in ovaries from treated rats is also reduced by up to 70% after a single 50 mg/kg dose and about 50% after 5 days treatment. In ether-stressed rats, the 3-ethyl analogue had no effect on plasma or adrenal concentrations of corticosterone after a single dose, and after 5 days of treatment corticosterone concentrations were suppressed to a smaller extent than in rats treated with AG [87]. The selectivity shown by these compounds *in vitro* is thus also demonstrable *in vivo*. The parent compound is currently undergoing toxicity tests in animals.

A novel series of compounds has been described [88] in which the glutarimide ring of AG is replaced by substituted or unsubstituted 3-azabicyclo[3.1.0]hexane-2,4-dione rings (8). The substituted analogues 1-(4-aminophenyl)-3-butyl-3-azabicyclo[3.1.0]hexane-2,4-dione and 1-(4-aminophenyl)-3-pentyl-3-azabicyclo[3.1.0]hexane-2,4-dione are potent inhibitors of aromatase *in vitro*, with K_i values of 20 nM or lower (cf. 180 nM for AG), and have no significant activity against CSCC. These compounds produce type II difference spectra with placental microsomes, suggesting that they bind to the aromatase enzyme in a manner similar to that of AG.

Two novel aromatase inhibitors, designated FCE24786 (*N*-cyclohexyl-2-(4-aminophenyl)propanamide (9)) and FCE24328 (cyclohexyl 2-(4-aminophenyl)propanoate) have been described by workers from Farmitalia Carbo Erba



(8) R= Bu or C_5H_{11}



(9)

[89]. These compounds are between 10- and 20-fold more potent against aromatase *in vitro* than AG, but have less effect against CSCC. Few details of these compounds are available to date.

Clinical use of aminoglutethimide

Of the compounds described above, only AG has yet been used therapeutically in humans. Originally developed as an anticonvulsant [90], it was subsequently reintroduced for the treatment of Cushing's syndrome after clinical reports of hypocortisolism in treated patients. Results were, however, disappointing and AG has proved more successful in the treatment of postmenopausal women with oestrogen-dependent breast cancer [91]. Conventional treatment regimens involve doses of 1,000 mg daily plus corticosteroid replacement, for example hydrocortisone 40 mg daily. Corticosteroid replacement is necessary due to the inhibition of adrenal steroidogenesis which, if uncorrected, would produce a reflex rise in the secretion of adrenocorticotrophic hormone (ACTH), and this would overcome the adrenal blockade [91]. Such regimens produce objective remission rates in approximately 33% of unselected patients [92, 93], although the response rate is increased in patients with oestrogen receptor-positive tumours [94] and in patients who have responded to other forms of endocrine therapy [92, 93]. Plasma oestrogen concentrations in treated patients are suppressed to approximately one-third to one-half pretreatment levels [93].

AG produces similar response rates to surgical ablative therapy such as adrenalectomy, and to tamoxifen (1) (a non-steroidal anti-oestrogen) therapy [92, 93]. Combination of AG with tamoxifen does not, however, produce a significant increase in response rate, and may be disadvantageous as the possibility of using secondary endocrine therapy after relapse is lost [95]. AG is of little use in the treatment of pre-menopausal breast cancer patients [96, 97], since it is relatively ineffective at inhibiting ovarian aromatase. This may be due to high ovarian concentrations of oestrogen precursors [97], and to increased pituitary gonadotrophin secretion resulting from blockade of oestrogen biosynthesis.

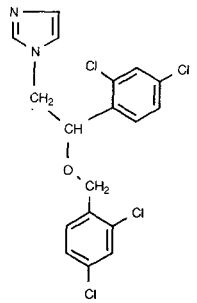
The major factor affecting the usefulness of AG is its toxicity. Side-effects are experienced by about 40% of patients [98], and necessitate discontinuation of treatment in about 5%. The most common side-effects include drowsiness, ataxia, morbilliform rashes, nausea and vomiting, and are usually transient, declining within 2–6 weeks. Rarer side-effects include depression and blood dyscrasias such as agranulocytosis [93]. The frequency of side-effects is related to the plasma concentrations of the drug; in one study 80% of patients with serum AG concentrations of 12 mg/l or above complained of

side-effects, while only 36% of patients with concentrations of 8 mg/l or less did so [99]. Due to its toxicity, AG is usually reserved for 'second-line' endocrine therapy in patients who have previously responded to tamoxifen. A recent study [100] found that toxicity is most pronounced in the elderly, and Plowman [101] has therefore concluded that AG is most appropriate only for younger post-menopausal women.

In an attempt to reduce the toxicity of AG, some workers [102, 103] have used low doses without corticosteroid replacement. Results have been contradictory; one group [102] has found that suppression of oestrogen is dose-dependent, while others [103] have found that a dose of 250 mg/day of AG alone was as effective as the standard dose of 1,000 mg plus 40 mg of hydrocortisone. Low-dose regimens have been reported to produce response rates similar to the conventional regimen [103], but other investigators [104] have concluded that the two regimens are not equally effective, and, moreover, that failure to replace glucocorticoid may present the risk of adrenal insufficiency.

Pharmacology of azole drugs as aromatase inhibitors

Imidazole-containing antifungal drugs such as miconazole (Daktarin®, (10)), clotrimazole (Canesten®, (11)) and ketoconazole (Nizoral®, (4)) are believed to act by inhibiting the fungal enzyme 14α-demethylase, which converts lanosterol to ergosterol [105]. The resulting accumulation of 14-methyl sterols appears to alter the fluidity of fungal cell membranes and may also alter the properties of membrane-bound enzymes [105].This enzyme is cytochrome *P*-450-dependent, and there is evidence [106] that the N-3 substituent of the azole ring of ketoconazole binds to the haem group in a manner comparable with the binding of AG to aromatase. It might be expected, therefore, that imidazole drugs could also bind to and inhibit mammalian cytochrome *P*-450-dependent enzymes. Early studies [107, 108] showed that imidazole drugs



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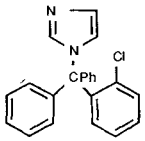

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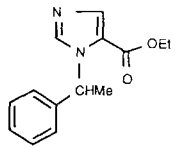


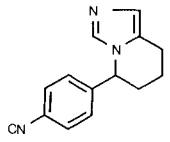
(12)

could inhibit mammalian enzymes at high concentrations, but this was not thought to be of any therapeutic significance [108]. In 1981, however, three cases of gynaecomastia were reported in male patients receiving treatment with ketoconazole for disseminated coccidioidomycosis [109], thus raising the possibility of an effect of ketoconazole on steroidogenic enzymes. Subsequently ketoconazole and other imidazole drugs, such as the antifungals, miconazole and clotrimazole, and the anaesthetic etomidate (12), have been shown to inhibit most cytochrome *P*-450-dependent steroidogenic enzymes both *in vitro* and *in vivo* [110–114]. Such enzymes include the $C_{17,20}$ lyase, which converts 17α-hydroxyprogesterone to androstenedione [112, 113], CSCC and 11-hydroxylase [62] and 6-, 16- and 16α-hydroxylases [111].

Inhibition of human aromatase by imidazole drugs has been extensively studied *in vitro* and *in vivo* [56, 115–120]. Ketoconazole has been most widely studied due to its widespread clinical use as an orally active broad-spectrum antimycotic and reports of associated gynaecomastia [109]. *In vitro*, it inhibits the aromatisation of both androstenedione [56] and testosterone [118], but is slightly less effective than AG (for example, K_i values with androstenedione as substrate are 6,000 nM for ketoconazole and 4,400 nM for AG [56]). In contrast to AG, ketoconazole has been reported to be a non-competitive inhibitor of aromatase [116], although inhibition of other steroid hydroxylases is apparently competitive in nature [114]; some other azoles such as miconazole have been reported to be competitive inhibitors [56].

Structure–activity relationships for the inhibition of human placental aromatase by imidazole drugs have been described by Nicholls and Shaw [118] and Ayub and Levell [119]. The most effective inhibitors, such as econazole (Ecostatin®), clotrimazole and miconazole, all possess an aralkyl substituent at position N1 of the imidazole ring. IC_{50} values for these compounds (testosterone as substrate) were 60 nM for econazole and clotrimazole and 160 nM for miconazole [118]. Ketoconazole was a relatively weaker inhibitor (IC_{50} value 36,000 nM) possibly due to the size of its aromatic side-chain, which could give rise to steric effects preventing the azole from binding effectively to the enzyme. Imidazoles bearing aliphatic substituents were ineffective inhibitors [118, 119], as were benzimidazole compounds such as midazolam (Hypnovel®) [118] and thiabendazole (Mintezol®) [119].

Since there is evidence [111] that imidazole drugs differ in their specificity towards different steroidogenic enzymes, these structure–activity studies raise the prospect of developing selective aromatase inhibitors based on the imidazole nucleus. One such drug, a serendipitous discovery, may be an interesting new compound, 4-(5,6,7,8-tetrahydroimidazo[1,5-*a*]pyridin-5-yl)-benzonitrile (CGS 16949A, (13)) (as the hydrochloride salt), which has been



(13)

described by workers from Ciba-Geigy [121, 122]. *In vitro* this compound inhibits aromatase competitively in both human placenta and rat ovary, being respectively 400- and 200-fold more potent than AG (IC_{50} values in human placenta of 5 nM for CGS 16949A and 1,900 nM for AG) [121]. In adult female rats, doses of 0.1–3.0 mg/kg reduced serum oestradiol concentrations [121]; the weight of the adrenals did not change, suggesting that inhibition of adrenal corticosteroid secretion (which would provoke a compensatory rise in ACTH secretion) did not take place. Repeated doses of 1.0–8.0 mg/kg daily for 42 days caused almost complete tumour regression in DMBA-treated rats [122], with no significant side-effects. In human mammary tumour cells *in vitro*, cell growth is inhibited by CGS 16949A at concentrations of 60–120μg/ml [122]. CGS16949A has been given to healthy human volunteers and been found to be effective and well-tolerated, but no details have been published to date.

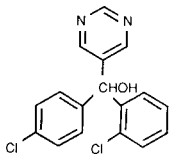
A substituted triazole, R151885 (1,1-di(4-fluorophenyl)-2-(1,2,4-triazol-1-yl)ethanol), has been shown to inhibit aromatase both *in vitro* and *in vivo* [123, 124] without apparently affecting other steroidogenic enzymes. Replacement of the triazole ring with an imidazole or pyridyl ring produces more potent inhibitors [124].

Clinical use of imidazole aromatase inhibitors

Of the array of imidazole drugs which have been shown to inhibit steroidogenesis *in vitro*, only ketoconazole has been used in the treatment of endocrine disease [125]. Due to its potent effect on $C_{17,20}$ lyase, it has ben used to reduce testosterone concentrations in patients with prostatic cancer [126, 127], but this has not proved successful due to problems of unacceptable toxicity and limited efficacy. Although it is an effective aromatase inhibitor *in vitro*, it has little effect on plasma oestrogens in treated subjects [128] and thus is of little potential use in the treatment of breast cancer. In one study [129], high doses (1,200 mg/day) produced partial remission lasting for over 8 months in one of three patients and stabilization of the disease in the remaining two. To date, however, the use of ketoconazole in hormone-dependent cancers has been disappointing.

Pyrimidine derivatives

Fenarimol (α-(2-chlorophenyl)-α-(4-chlorophenyl)-5-pyrimidinemethanol, (14)) is a pyrimidine carbinol fungicide which, like the imidazole antimycotics, acts by inhibiting ergosterol biosynthesis [130]. When administered orally to male rats, it causes a dose-dependent reduction in fertility which is apparently due to a decrease in sexual behaviour [131]. This effect is particularly marked in the male progeny of female rats treated with fenarimol during pregnancy, since it is concentrated in the milk, reaching concentrations between 3- and 5-times higher than those found in maternal plasma [131], and thus is readily available to the neonate during the prenatal period. Since there is evidence that in the male rat sexual behaviour depends on exposure of the brain to androgens, and subsequent aromatization of androgens to oestrogens, during the perinatal period [132], these findings suggest that the infertility produced by fenarimol may be due to inhibition of aromatization within the central nervous system. Subsequent experiments [133] showed that fenarimol was concentrated in the brain of neonatal rats, and that it inhibited the formation of [^{3}H]oestrogens in the brain after administration of [^{3}H]testosterone. Furthermore, fenarimol also inhibited the aromatase activity of rat ovaries *in vitro*, with an IC_{50} value of 4,100 nM [133]; in the same system AG had an IC_{50} value of 260 nM.

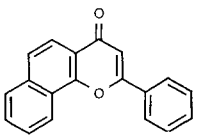


(14)

Fenarimol is thus a relatively weak inhibitor of aromatase *in vitro*. Consequently, a series of 5-substituted pyrimidine derivatives have been evaluated as aromatase inhibitors [134]. The most effective inhibitors were diarylpyrimidine methanols and diarylpyrimidine methanes containing electron-withdrawing substituents, especially chlorine atoms, at positions 4- and 4′. The most potent, α-(4-chlorophenyl)-α-(4′-chlorophenyl)-5-pyrimidinemethane (5-[bis-(4-chlorophenyl)methyl]pyrimidine, LY56110, had an IC_{50} value of 55 nM in PMSG-treated rat ovaries [134]. This compound therefore appears to be a potentially useful aromatase inhibitor, but few other details have been published to date.

Flavones and related compounds

Flavonoids are 2-phenyl-1-benzopyran-4-one derivatives which are widely distributed throughout the plant kingdom. Some synthetic flavonoids, notably the 7,8-benzoflavones (α-naphthoflavones (15), IUPAC name: 2-arylnaphtho-[1,2-*b*]pyran-4-ones), chrysin and apigenin, have been shown to act as potent competitive inhibitors of human placental aromatase [135, 136]. In this system, 7,8-benzoflavone and chrysin have IC_{50} values of 70 and 500 nM respectively (androstenedione as substrate). Hydroxylated metabolites of 7,8-benzoflavone, with substituents at positions 6,7,8,9 and 10 are also potent inhibitors, the most potent being the 9-hydroxy derivative (IC_{50} value of 20 nM) [137]. The latter compound has a K_i value of 5 nM, which indicates that it binds to the enzyme more avidly than either androstenedione or testosterone (K_m values 10 nM and 80 nM respectively) [137] and it has therefore been proposed that the 7,8-benzoflavone ring system, which is of similar dimensions to the steroid nucleus, binds to the steroid-binding site of the enzyme. To date, however, this inhibitory property has not been exploited therapeutically.



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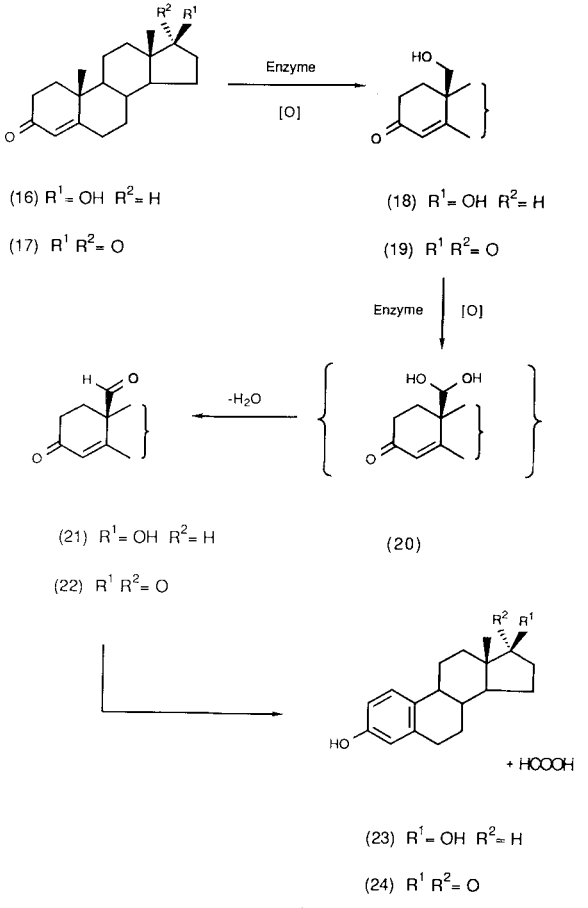
Other non-steroidal aromatase inhibitors

Miscellaneous compounds of pharmacological interest have been shown to inhibit aromatase *in vitro*, including the antimalarial drug mefloquine, [138], the oral hypoglycaemic agent, tolbutamide [139], and nicotine, its metabolite cotinine, and anabasine, all of which are found in tobacco [140]. These effects probably have little clinical relevance, although the latter three compounds may account for the reduced urinary [141] and plasma [142] oestrogen concentrations found in smokers [140].

MECHANISM OF ACTION OF AROMATASE

The accepted pathway for the biosynthesis of the oestrogens from the appropriate androgen substrate is shown in *Scheme 2.1.* The conversion of either testosterone (16) or androstenedione (17) to their respective oestrogens, oestradiol and oestrone, is catalysed by a *P*-450-NADPH-flavoprotein-reductase-dependent enzyme complex, aromatase (AR) [143, 144]. The overall



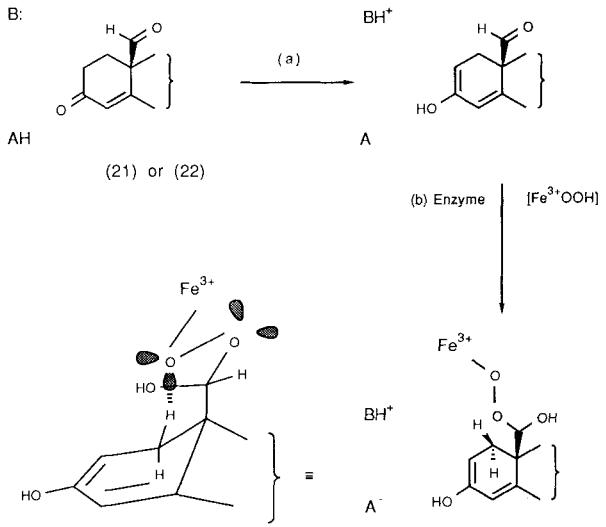
Scheme 2.1. Generally accepted biosynthetic pathway for the oestrogens. (16) Common name, testosterone; formal name, 4-androsten-17β-ol-3-one. (17) Common name, androstenedione; formal name, 4-androstene-3,17-dione.

oxidative 10-demethylation of the substrate requires three equivalents of NADPH and molecular oxygen [144]. The 10-methyl group of the androgen is monohydroxylated to intermediate ((18) or (19)), which is further hydroxylated stereoselectively; this occurs at the 19-pro*R* carbon–hydrogen bond to generate a *gem*-diol (20) which spontaneously dehydrates to yield the aldehyde ((21) or (22)) [145, 146]. Evidence for the intermediacy of (18) and (19), and (21) and (22) has been presented [147, 148]. The position of the third hydroxylation, that is, at positions 1- or 2-, and the stereochemistry of the

elimination of the 1- and 2-hydrogens are still actively disputed. It has been demonstrated, however, that the enzyme discriminates between the inserted oxygens [148]. Oxygen inserted in the first and third steps is retained in the formate generated on aromatization. The mechanistic implications of this observation have led to proposed hypothetical pathways to explain the eventual aromatization step. Two of these pathways involve the hemiacetal formation between (i) a 1-equatorial hydroxy [149] or (ii) a 2-axial hydroxy group and the 19-aldehyde [150]. A third proposal invokes a peroxy intermediate, the rearrangement of which accounts for the observed stereochemical outcome of the reaction [151, 152]. Two excellent reviews of the mechanism of AR presenting the evidence for the third hydroxylation have been published [153, 154].

A recent communication, however, from Cole and Robinson suggests that the androgen substrates are dihydroxylated stepwise to form the 19-aldehyde. This aldehyde is then attacked by an iron-bound hydroperoxy group to give an intermediate hydroperoxy acetal which then collapses to generate the aromatized product (see *Scheme 2.2*) and formic acid [155].

It would be easy to visualize the enzyme binding the substrate molecule, and an amino acid that recognizes the 3-carbonyl via hydrogen bonding



Scheme 2.2. Proposed mechanism for the aromatisation of the 19-aldehyde intermediate via a hydroperoxy acetal. (a) Enolization step; (b) enzyme-assisted nucleophilic attack by hydroperoxyl intermediate. AH/A^-, acidic amino-acid residue and conjugate base. B/BH^+, basic amino-acid residue and conjugate acid.

donates a proton to the 3-position oxygen, which allows the *in situ* generation of an enolic form; the proton at position 2 is then accepted by a basic amino-acid residue located nearby. This process would generate a dienyl transitional state, which, on the third and final attack of an iron-bound oxygen species (possibly a hydroperoxide) on the 19-position, drives an elimination resulting in the formation of the oestrogen and formic acid. An examination of a Dreiding model of this possible final intermediate indicates that its collapse may occur via a six-membered 'chair like' cyclic transition state. This transition state could be invoked whether an electron pair or radical mechanism is chosen (*Scheme 2.2*).

INHIBITION OF AROMATASE BY STEROIDS

Approaches believed to interfere with the catalytic processes of AR fall into three main categories:

(a) competitive inhibition (reversible);

(b) mechanistic inhibition (reversible) – inhibitors that may co-ordinate to the haem iron;

(c) mechanistic inhibition (irreversible) – inhibitors that are metabolized to yield reactive alkylating species at the enzyme site.

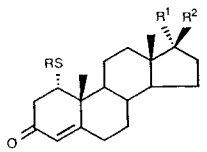
It must be stated that the above groups are a convenience for 'historical classification'. Design of inhibitors in one of the three categories has often meant contribution of knowledge to the other two and the increase in understanding of the active site of AR allows the more rational design of inhibitors targetted to each category.

The information available on steroidal inhibitors of AR has been collected from diverse sources. In the past 10–15 years, biochemists, endocrinologists and, more recently, medicinal chemists have all investigated the characteristics of the enzyme for their various reasons, and as such, non-standardization of the techniques used to evaluate inhibition has presented a major problem in analysis of the data. Data vary from qualitative to quantitative. IC_{50} and K_i values are quoted interchangably over differing substrate concentrations (with either substrate) and quality of enzyme preparation and without the use of internal standard inhibitors as reference points. Fortuitously, the enzyme generally has been obtained from the same tissue source, human placenta. If a concerted effort to manipulate the aromatase enzyme for therapeutic benefit is to succeed, standardization of biochemical procedures would enhance greatly the medicinal chemist's understanding of the structural requirements for the enzyme active site and aid in the design of new more effective inhibitors.

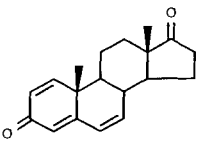
1- AND 2-SUBSTITUTED SUBSTRATE ANALOGUES

Synthesis of 1α-substituted thioethers (25) has led to the 1α-phenylthio, benzylthio and 4-diethylaminobenzyl compounds [156]; all exhibited poor competitive inhibition of AR. It was concluded that "functionality in this region was expected to interfere with appropriate interaction of the substrate at the active site" [156].

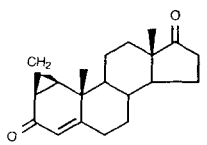
The disclosure by Schering of an irreversible inhibitor Sh489, 1-methylandrost-1,4-diene-3,17-dione does, however, suggest a small degree of tolerance in this region [157]. It may be that the 1-methyl substituent is acceptable due to its planarity with the steroid ring system, whereas the conformational analysis of the bulky substituents in the 1α-thioethers indicate probable orthogonality to the plane. There appears to be a lack of information on bulk tolerance in the 1- and 2-regions and in particular it would be of interest to see how β-substitution affects substrate binding. One can speculate on the irreversible nature of Sh489 being due to the radical attack of an activated iron-oxygen complex on the 1,2-double bond generating a reactive radical species which in turn covalently binds to the haem, thereby deactivating the catalytic site. This method of deactivation of *P*-450 enzymes has been demonstrated in hepatic microsome preparations and is referred to as prosthetic haem alkylation [158]. Another possibility is that two steps of the catalytic process can occur, that is, generation of the 19-aldehyde intermediate and that the third step requires covalent binding to the 19-position followed by the abstraction of a hydrogen atom from the 1-position, which in the natural substrate is available, but in Sh489 is missing. A demethyl analogue of Sh489,



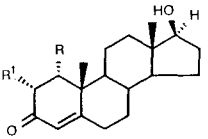
(25)



(26)



(27)



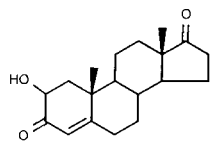
(28, R=Me_3Si , R^1=H)

(29, R=H , R^1= Me_3SiCH_2)

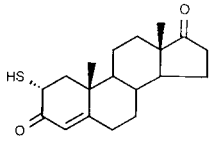
1,4,6-androstatriene-3,17-dione (26) was initially shown to be a reversible inhibitor of AR [159] (with an apparent K_i = 180 nM [160]), but later work showed that the compound could irreversibly deactivate the enzyme exhibiting a $k_{inact} = 1.1 \times 10^{-3}\ s^{-1}$; this would presumably act by a similar mechanism.

Schering also produced a 1,2-β-methylene substituted androstenedione analogue (27) which was patented as a compound capable of lowering oestradiol levels [161], along with other substituted 1-alkylandrosta-1,4-diene- and -1,4,6-triene-3,17-dione derivatives [162, 163].

The synthesis of 1α- and 2α-trimethylsilated derivatives of testosterone was undertaken with the intention of producing enzyme-activated inhibitors [164]. Both the 17β-hydroxy-1α-(trimethylsilyl)- (28) and 17β-hydroxy-2α-[(trimethylsilyl)methyl]- (29) androst-4-en-3-ones proved to be inactive. This lack of activity may, however, be a reflection of the inherent low affinity of the 17β-hydroxy as compared with those of 17-keto androgens (that is, testosterone analogues in comparison with androstenedione) as AR substrates as well as an indication of lack of bulk tolerance [159]. Both the 2-hydroxyandrost-4-ene-3,17-dione (30) and the 2-hydroxyandrost-2,4-diene-3,17-dione were found to be inactive against AR [165]. It has been reported, however, that 2α-mercaptoandrost-4-ene-3,17-dione (31) has potent suicide inhibitory activity against AR, although no quantitative data have been presented [166].



(30)

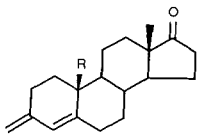


(31)

3-SUBSTITUTED SUBSTRATE ANALOGUES

Removal or reduction of the 3-keto group to either of the epimeric alcohols of the substrate results in a drastic loss of affinity for the enzyme [159].

Conversion of known substrates of AR to their 3-methylene derivatives resulted in competitive inhibitors, the most effective of which were the 17-keto analogues 3-methylene- (32), 19-hydroxy-3-methylene- (33) and 3-methylene-19-oxo- (34) androst-4-en-17-ones, with apparent K_i values of 4.7, 13 and 24 nM, respectively [167]. A parallel series of 17-hydroxy analogues exhibited lower potency, supporting the general observation that the 17-keto compounds demonstrate higher affinity for the enzyme.



(32) R= Me (35) R= CHF_2

(33) R= CH_2OH (36) R= CHC≡CH

(34) R= CHO

It was noted that the 19-hydroxy- (33) and 19-oxo-3-methylene (34) analogues exhibited time-dependent irreversible inhibition of AR while the corresponding 10-methyl did not, suggesting that functionalization of the 10-methyl was necessary for the enzymatic activated inhibition.

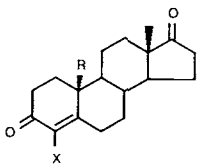
From a further observation that the 3-methylene analogues (35) and (36) of 10β-difluoromethyl- and 10β-(2-propynyl)estr-4-ene-3,17-dione, both known to be irreversible inhibitors of AR [168, 169], were inactive, it was concluded that the 3-keto function was required to assist in the primary oxidation of the 10-methyl group of these inhibitors. This conclusion indicates a possible enzyme guided movement of the primary oxidation products to realign for the 2-oxidation leading to eventual aromatization, assuming this pathway is being evoked. This is consistent with the observation that the 3-methylene-19-hydroxy (33) and 19-oxo (34) analogues are activated, whereas the 10-methyl is not. If one accepts the postulate that hydroxylation occurs via a rebound mechanism [170], that is, a primary abstraction of the hydrogen at the site to be converted by an activated iron-oxygen species and the transfer of a hydroxyl radical from this species back to the carbon-centred radical to form a C-OH bond, the irreversible nature of (33) and (34) is explicable. The radical generated by abstraction of the 2-hydrogen in the 3-methylene analogues (33) and (34) would lead to an allylic system that may allow a redistribution of the electron density to form a radical that could react elsewhere in the active site, for example, at the site recognizing the 3-keto function of the substrate.

The formation of a hydroperoxy acetal intermediate (see *Scheme 2.2*) may provide an explanation for the irreversibility of these inhibitors. The covalent linking of an iron-bound hydroperoxy group to the 19-position may occur, but the resulting cleavage of this intermediate may not be possible due to the absence of the correct transitional state leading to an irreversibly bound complex.

4-SUBSTITUTED SUBSTRATE ANALOGUES

The chemistry and biochemistry of 4-substituted substrate analogues

4-Hydroxyandrostene-3,17-dione ((37), 4-OHA) is currently used in the clinical treatment of oestrogen-sensitive breast cancer [171]. This compound and its analogues have been the subject of extensive investigation as steroidal inhibitors of AR [165]. 4-OHA is an irreversible inhibitor of AR with reasonable affinity for the enzyme, the apparent K_i being 170 nM [172]. 4-OHA itself is rapidly metabolized and cleared primarily as the glucuronide conjugate, in rat and Rhesus monkey [173], so that much research has been undertaken to alter the pharmacokinetics and thus improve the adverse metabolic clearance. Conversion of the hydroxy group to esters (38)–(41), particularly with the more bulky analogues (39)–(41), reduces the activity. It was found that activity was generally maintained when the 4-OHA nucleus was further conjugated to the 1,4-diene (48), 4,6-diene (46) and 1,4,6-triene (47) derivatives [165]. The irreversibility of the inhibitors was retained in these analogues but it was found that inactivation of the enzyme occurred more rapidly with (46, $t_{1/2}$ = 6–8 min) as compared with 4-OHA ($t_{1/2}$ = 20 min). Two analogues of 4-OHA have been synthesized without the 10-methyl group. Both 4-acetoxy- (49) and 4-hydroxy-4-estrene-3,17-dione (50) showed competitive reversible inhibition of AR. We suggest that the lack of irreversible action of these derivatives is due to the necessity for oxidation of the 10-methyl group to activate the substrate analogues for covalent binding to the apoprotein.



(37) R= Me X= OH

(38) R= Me X= OAc

(39) R= Me X= OCOPh

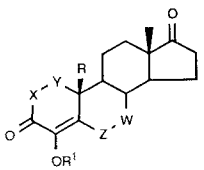
(40) R= Me X= $OCO(CH_2)_2COOH$

(41) R= Me X= $OCO(CH_2)_{10}Me$

(42) R= Me X= F

(43) R= H X= F

(44) R= CHF_2 X= OH



(45) X=Y= CH R= Me R^1= H

(46) X=Y= CH_2 W=Z= CH R= Me R^1= H

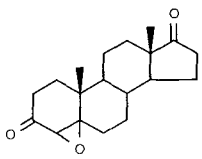
(47) X=Y=W=Z= CH R= Me R^1= H

(48) X=Y= CH W=Z= CH_2 R= H R^1= H

(49) X=Y=W=Z= CH_2 R= H R^1= Ac

(50) X=Y=W=Z= CH_2 R= H R^1= H

Without the 4-hydroxy group, the 4,6-diene (46) and 1,4-diene (48) show weak activity. The 4-thio isosteres of 4-acetoxy- and 4-hydroxyandrostenedione were less active, as were the 4-chloro and 4-methoxy analogues. The 4α,5α-(51) and 4β,5β-(52) epoxides were fairly potent, despite the disruption of the 3-keto-4-ene conjugation.



(51) 4α,5α-epoxy

(52) 4β,5β-epoxy

A series of 4-thioethers were synthesized as substrate analogues of androstenedione with alkyl, aralkyl and aryl side-chains. The K_i values of this series varied from 36 to 73 nM (K_m (androstenedione) = 53 nM) and allowed a structure–activity relationship to be determined for the 4-thioethers. A delineation of the available volume around this region of the substrate was proposed as a tight enzyme pocket that can accommodate substituents up to 5.5 Å in length [174].

A recent attempt to overcome the unfavourable metabolism of 4-OHA has resulted in the synthesis of the 4-fluoro- (42) and 19-nor-4-fluoroandrostenediones (43) [175]. These compounds show reasonable affinity for the enzyme, K_i = 90 and 15 nM respectively, and were found not to be irreversible but were shown to be androgenic [175]. In an attempt to block metabolism of the 10-methyl of 4-OHA the 19,19-difluoro analogue (44) was synthesized but was found to be less potent – *in vitro* IC_{50} value 3,300 nM (*cf.* 4-OHA IC_{50} value 200 nM) [176].

4-Amino analogues of 4-OHA and its unsaturated derivatives have been synthesized by Farmitalia and were shown to be irreversible inhibitors [177]. 4-Mercapto-6-substituted androstenediones have also been described in a recent Farmitalia patent [178], although no details are available on the biological activity.

Clinical pharmacology of 4-hydroxyandrostenedione

In laboratory experiments, it was found that 4-OHA reduced plasma oestrogen concentrations and inhibited ovarian and peripheral aromatase activity

in PMSG-treated rats [179, 180], and produced tumour regression in DMBA-treated rats [181]. It was therefore subjected to preliminary clinical trials to establish its efficacy in humans. In the first trial [182, 183], 11 patients with metastatic breast cancer received 500 mg by intramuscular injection every 7 or 14 days. Four patients showed objective responses, with healing of bone metastases and reduction in size of soft tissue metastases, and a fifth patient showed stabilization of the disease. One of the patients who responded subsequently relapsed after 4 months. Plasma oestradiol concentrations were measured in 5 patients, and a significant suppression was observed after 24 h; this was maintained for up to 7 days, concentrations after 6 days of treatment being approximately 46% of baseline values [91, 182]. The only side-effects reported were local pain at the injection site in three patients, and hot flushes in two. Subsequent studies [184] showed that maximal suppression of plasma oestradiol is achieved within the first week of treatment, and that effective doses are less than the 500 mg used in the earlier trial. Despite undergoing extensive glucuronidation during its first pass through the liver [185], 4-OHA is active when given orally [184]. Oral doses of 250 mg reduce plasma oestradiol by about 53% [185], and doses of up to 1,000 mg daily produce little further suppression. It was therefore proposed [186] that further trials should use maximum doses of 250 mg/day when given orally and 250 mg/2 weeks when given by intramuscular injection. A recent trial [187] has examined the response to 4-OHA in women with metastatic breast cancer who received either weekly doses of 500 mg i.m. or daily doses of 250 mg orally. Response rates after 3 months of treatment were 27% (14/58 patients) for the parenteral administration and 26% (8/31) for oral administration. Side-effects were associated mainly with parenteral injection; the only serious side-effect after oral administration was the development of leucopoenia in a single patient. Trials are continuing to determine optimal doses and routes of administration.

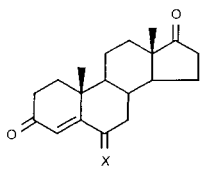
In an attempt to improve the bioavailability of oral 4-OHA, an analogue has been developed [188] in which the 4-hydroxy group is replaced by a fluorine atom (4-fluoroandrostenedione). This compound has similar potency against aromatase, but is less extensively metabolized than 4-OHA. In isolated rat hepatocytes, glucuronidation of this compound is reduced by about 15% compared with 4-OHA [189]. An alternative approach to improving the bioavailability of 4-OHA involves concomitant administration of valproate [189]. In rats, this produces a dose-related reduction in glucuronidation *in vivo*, but it has not yet been used in clinical trials.

6-SUBSTITUTED SUBSTRATE ANALOGUES

The potential for the 6-substituted androgens to act as probes of the AR active site has yet to be fulfilled. Few derivatives have been synthesized that contribute any significant increase in knowledge about the accessible volume of the enzyme active site capable of being utilized that may assist in the design of inhibitors with increased potency.

Several 6-oxygenated derivatives have shown an order of preference for the AR enzyme. The 6-oxo (53), β-hydroxy (54), β-hydroperoxy (55), α-hydroperoxy (56) show K_i values that decrease in the above order, giving values of 250, 500, 650 and 750 nM, respectively. Both the α- and β-hydroperoxy derivatives were substrates for AR giving K_m values of 280 and 250 nM, respectively (K_m (androstenedione) = 200 nM) [190]. Further to this work was a report that the 6-hydroperoxy derivatives (55) and (56) exhibited irreversible inactivation of AR and that the time-dependent inactivation did not require the presence of NADPH. The irreversibility was therefore due to the inherent reactivity of the substrate analogues and did not depend on enzyme activity. The authors suggested that the hydroperoxy groups were reacting with neighbouring cysteine residues in the AR active site [38].

4-Androstene-3,6,17-trione has been described as an irreversible inhibitor of AR [191] with a range of apparent K_i values 1250 [192], 430 [160] and 250 nM [165]. The work that reported an apparent K_i = 430 nM also recorded a $k_{inact} = 4.03 \times 10^{-3}\ s^{-1}$. The group reporting the inhibition at a K_i of 1,250 nM also reported the reduced product 3β-hydroxyandrostene-17-one to be a less potent irreversible inhibitor, with a K_i of 6,250 nM; both compounds demonstrate competitive inhibition [192].



(53) X=O

(54) X= α-H β-OH

(55) X= α-H β-OOH

(56) X= β-H α-OOH

(57) X= β-H α-Br

(58) X= β-Br α-H

(59) X= α-H β-F

(60) X= α-H β-F 2β-F

Interesting results have been obtained with the 6α- (57) and 6β- (58) bromoandrostenediones, compounds which were originally synthesized as potential affinity ligands for the enzyme [193]. The 6α-bromo compound appears to be a competitive inhibitor with respect to androstenedione (K_i = 3.4 nM), whereas the 6β-bromo isomer is an enzyme-activated irreversible inhibitor (apparent K_i = 800 nM, k_{inact} = 0.025 min^{-1}) [194]. Although there are conflicting results over which isomer's inhibition is time-dependent [195], in the authors' opinion the former report seems to indicate the true assignment [194]. The inactivation of AR, in an irreversible manner, is not readily explicable in these compounds and needs verification with further consideration as to possible mechanisms.

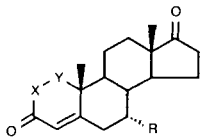
In an attempt to synthesize 4-OHA (37) and its corresponding Δ-6 derivative, two 6-fluorinated substrate analogues were prepared and screened for AR activity. The action of Olah's reagent on (37) in the presence of mercury(II) oxide led to the formation of 6β-fluoroandrost-4-ene-3,17-dione (59); androsta-4,6-diene-3,17-dione could be prepared if tetrabutylammonium fluoride was used instead [196]. However, when an alternative route was chosen by these authors as the method of choice for preparing (43), it was discovered that 2α-hydroxyandrost-4-ene-3,17-dione when treated with Olah's reagent in dichloromethane yields (59). It was also found that treatment of 4β,5β-epoxy derivative (49) led to the formation of 2β,6β-difluoroandrost-4-ene-3,17-dione (60). Both the 6β-fluoro- (59) and 2β,6β-difluoro- (60) derivatives were active against AR; (59) was reported to have excellent activity, whereas (60) was described as weak (no data) [196].

A recent report of 6-substituted androst-4-ene-3,17-dione analogues synthesized by Shionogi has been released. Details are lacking but suggest that the 6-azido, -thiocyanato, and propargyloxy compound (no stereochemical details) are potent competitive inhibitors of AR and that unspecified examples were shown to significantly reduce ovarian oestrogen secretion in the rat [157].

7-SUBSTITUTED SUBSTRATE ANALOGUES

The same group that investigated the structure–activity relationships (SAR) of the 1-(thioether) androstenedione derivatives (see section on 1- and 2-substituted substrate analogues) have also explored the substitution of androstenedione in the 7-axial position [156]. A series of 7α-thioalkyl, -thioaralkyl and -thioaryl androstenediones proved to be a successful probe of the active site and resulted iin the synthesis of one of the most potent competitive inhibitors of AR.

7α-(4′-Aminophenyl)thioandrost-4-ene-3,17-dione (61) exhibits an apparent K_i of 18 nM and the activity within the series of compounds tested ranged from 18 to 69 nM [197]. These results suggest a high degree of bulk tolerance in this region of the enzyme substrate binding site. This view is supported by work performed on 7α-thioalkyltestosterone derivatives. These compounds were primarily synthesized as androgens capable of affinity-labelling hormonal receptor sites but were also tested against AR using both androgens as substrates [198]. Again, these workers found that the 17-keto compounds were more potent than the corresponding alcohols as inhibitors of AR. The enzyme was found to tolerate at least the bulk of a hydroxypropyl group at the 7α-position. It was found that the affinity for the enzyme decreased in the order 7α-ethyl, -propyl and -butyl for the testosterone analogues.



(61) R= 4-$H_2NC_6H_4S$ X=Y= CH_2

(62) R= 4-$H_2NC_6H_4S$ X=Y= CH

The synthesis of the 7α-alkyltestosterone derivatives was achieved by the conjugate addition of the appropriate Grignard or lithium dialkyl copper reagent to 17α-hydroxyandrost-4,6-diene-3-one propionate followed by hydrolysis of the ester.

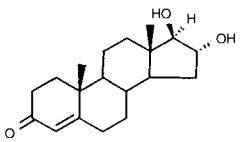
The route developed by this group would allow the synthesis of higher and branched-chain homologues that would permit extensive probing of the conformational requirements of the substrate binding site in this region of the steroid. The synthetic route used, however, seems only to have produced the 7α-isomers. It would be of great value in the determination of a full SAR picture to include the corresponding 7β-isomers, and a report included in this reference suggested that a variety of lithium dialkyl copper compounds did form mixtures of the isomers on addition to steroidal 4,6-diene-3-ones [199].

Further work on the 7α-(4′-aminophenyl)thioandrostenedione (61, 7-APTA) was undertaken. The synthesis of an unsaturated derivative of (61) has resulted in the conversion of a reversible competitive inhibitor of AR into an enzyme-activated irreversible inhibitor. 7α-(4′-Aminophenyl)thio-1,4-androstadiene-3,17-dione (62, 7-APTADD) has an apparent K_I value of 9.9 ± 1.0 nM (K_m for androstenedione of 52.5 ± 5.9 nM) and demonstrated rapid

enzyme deactivation kinetics $t_{1/2} = 1.38 \pm 0.92$ min, the most rapid reported to date [199].

16-SUBSTITUTED SUBSTRATE ANALOGUES

A single report of the action of 16-substituted substrate analogues on AR has been found. 16α-Hydroxytestosterone (63) was shown to be a substrate for AR being metabolizeed to oestriol, but it also inhibited (K_i = 7,000 nM) the aromatization of androstenedione (K_m = 95 nM). The aromatization of (63) was shown to be inhibited completely by metyrapone but not by carbon monoxide, whereas the aromatization of testosterone (16) was inhibited by both. The 16α-hydroxytestosterone aromatization was strongly inhibited in a competitive manner by androstenedione (17), while it appears that (63) had essentially no effect on the conversion of (17). At very high concentrations of (63) and a subsaturating concentration of (17), however, (63) appeared to compete non-competitively with (17) for the enzyme. The authors concluded that the evidence suggested the presence of two aromatase *P*-450 enzymes in human placenta. However, an alternative explanation may be that 16α-hydroxytestosterone (63) occupies a secondary binding site as well as being capable of occupying the substrate binding site at the active site [200]. Further studies on the mechanistic aspect of the biosynthesis of (63) to oestriol have been carried out, demonstrating that the 19-hydroxy and 19-oxo derivatives of (63) are involved in the biosynthetic pathway and that the conversion involves the same enzyme and enzymatic processes [201].

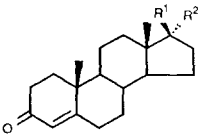


(63)

17-SUBSTITUTED SUBSTRATE ANALOGUES

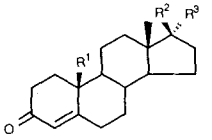
Early work on the inhibition of AR and its affinity labelling led to the synthesis of 17β-bromoacetylamino-4-androsten-3-one (64) from the corresponding 17β-amino derivative by condensation with bromoacetic acid in the presence of DCC (dicyclohexylcarbodiimide). This derivative along with two other potential affinity labels, the 16α-bromoacetoxy-4-androstene-3,17-dione and

16α-bromoacetoxy-4-androstene-3,6,17-trione were reported to act as competitive inhibitors although the activity (no quantitative data) was weaker than a third compound, 17β-bromoacetoxy-4-androsten-3-one (65) [202]. No claim for their irreversibility towards AR was made.



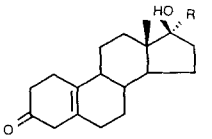
(64) R^1 = $NHCOCH_2Br$ R^2 = H

(65) R^1 = $COCH_2Br$ R^2 = H



(66) R^1 = OOH R^2 = OH R^3 = C≡CH

(67) R^1 = H R^2 = OH R^3 = C≡CH



(68) R = C≡CH

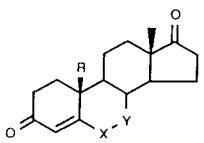
The inhibition of human placental aromatase has been studied with a series of 17α-ethynyl-substituted 10β-hydroperoxy- and related 19-nor-steroids [203]. 17α-Ethynyl-10β-hydroperoxy-17β-hydroxy-4-estren-3-one, Sch10015 (66) was demonstrated to inactivate AR in a time-dependent manner (K_i = 41,000 nM, $t_{1/2}$ = 20 min, K_m (androstenedione) = 90 nM). Sch10015 has been shown to be a potent orally active contraceptive agent in rats and rabbits and is the product of aerial oxidation of a solution of norethynodrel, also a known contraceptive (67). Norethynodrel and another contraceptive steroid, norethisterone (68), were screened against AR and exhibited only competitive reversible inhibition (K_i = 38,000 and 48,000 nM, respectively) with low affinity for the enzyme. The Schering compound was thought to deactivate AR by the same mechanism as 10β-hydroperoxy-4-estrene-3,17-dione (92), although, in contrast to (66) (see p. 288), it acted in the presence or absence of NADPH *in vitro*. This difference in activity was attributed to the different positioning of the substrate analogues in the active site. The affinity of (92) for the enzyme is *ca.* 100-times that of (66); this was taken to confirm the plausibility of this explanation. Since the other derivatives did not exhibit irreversible inhibition, it was considered that conversion of (67) and (68) to (66) did not occur readily in the microsomal preparation. It was speculated that the observed potent contraceptive effect of Sch10015 may be due to the irreversible inhibition of oestrogen biosynthesis.

10- AND 19-SUBSTITUTED SUBSTRATE ANALOGUES

Substitution at the 10-position has been extensively investigated both by biochemists and medicinal chemists, endeavouring to impart inhibitory activi-

ty and help elucidate the mechanism of action of the enzyme. Both groups have contributed much towards the knowledge of the active site of AR. The work has been centred mainly around manipulating the catalytic action of the AR enzyme, since the 10–19 bond is of obvious importance and substrate analogues in both the (b) and (c) categories (see p. 272) have been developed.

The proposed intermediates in the biosynthetic pathway of the oestrogens (see *Scheme 2.1*), the 19-hydroxy- and 19-oxo-androgens, act as competitive inhibitors of AR and have been the subject of considerable investigation [12]. An academic approach to the role of substrate conformation in the determination of the stereospecific nature of the enzyme process has investigated the necessity for a particular conformation of the 19-hydroxymethyl group in (19-OHA, (19)). In this work, analogues of (69), diastereoisomeric 19-hydroxyandrosta-4,6-diene-3, 17-dione analogues (70) and (71) were synthesized and were shown to be suicide inactivators of AR (K_i = 4,800 nM, $k_{inact} = 1.77 \times 10^{-3}\ s^{-1}$ (*S* diastereoisomer) and K_i = 3,200 nM, $k_{inact} = 3.42 \times 10^{-3}\ s^{-1}$ (*R* diastereoisomer) [154]. This is in contrast to that found with the reduced substrate analogues (72) and (73), the *R* diastereoisomer (72) being only a competitive inhibitor, the *S* (73) a suicide substrate [204]. The conclusion from the study is that the steric constraints intrinsic to 19-hydroxyandrost-4-ene-3,17-dione analogues cannot be a major determinant in the stereospecificity of the enzymic oxidation by AR.



(69) R= CH_2OH X=Y= CH

(70) R= (1R)-1-hydroxy-2-propynyl X=Y= CH

(71) R= (1S)-1-hydroxy-2-propynyl X=Y= CH

(72) R= (1R)-1-hydroxy-2-propynyl X=Y= CH_2

(73) R= (1S)-1-hydroxy-2-propynyl X=Y= CH_2

(74) R= Et X=Y= CH_2

(75) R= (1S)-1-hydroxyethyl X=Y= CH_2

(76) R= (1R)-1-hydroxyethyl X=Y= CH_2

(77) R= COMe X=Y= CH_2

(78) R= H X=Y= CH_2

The effect of substituting the substrate steroid, androstenedione, and the 19-OHA (19) and 19-oxo-A (22) steroids has been investigated. The appropriate homologues extended by a methyl group have been synthesized and evaluated both as possible substrates and inhibitors of AR. It was found that

10-ethylestr-4-ene-3,17-dione (74) acted as a competitive inhibitor (K_i = 81 nM) with affinity comparable with that of androstenedione (K_m = 153 nM). The two diastereoisomeric alcohols, 10-[(1*S*)-1-hydroxyethyl]- (75) and 10-[(1*R*)-1-hydroxyethyl]- (76) estr-4-ene-3,17-dione, were similar to one another in their affinity for AR (K_i = 11,000 nM and 9,900 nM, respectively; K_m (androstenedione) 64 nM) but exhibited *ca.* 50-fold less affinity than 19-OHA (K_i = 210 nM [154]).

The 19-oxo-A (22) has been reported to have a similar affinity for AR (K_i = 260 nM) as 19-OHA [205]. 10-Acetylestr-4-ene-3,17-dione (77) was found to bind *ca.* 65-times more tightly to AR (K_i = 150 nM, K_m(androstenedione) = 89 nM) than did the alcohols (75) and (76).

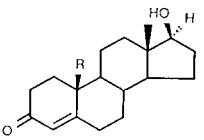
It was found that substitution of a methylene group in 19-OHA, to give (75) and (76) prevented the first and third hydroxylations carried out by AR. The diastereoisomeric alcohols (75) and (76) were converted by AR to the 10-acetyl derivative (77) by the enzyme. The 10-acetyl derivative (77), however, was not metabolized by AR to oestrone. This work concluded with the statement that the presence of a 19-methyl group in these substrate analogues does not alter the regiospecificity of AR. The first and third hydroxylations are selectively blocked, while the second proceeds with loss of stereospecificity [154].

Both 19-norandrostenedione (78) and androstenedione (17) are known substrates for AR. Kinetic evidence suggests that they share a common catalytic site on the enzyme. 19-Norandrostenedione is a competitive inhibitor of the aromatization of (17); the K_i for its inhibition (120 nM) is similar to the K_m for its metabolism (132 nM). The two substrates differ in their sensitivity towards inhibition by cyanide of AR. Spectroscopic studies indicate that (17) competes with cyanide, whereas the 19-nor derivative seems to promote binding of cyanide to the haem-iron [206]. A model for the active site of AR was proposed from analysis of these data, suggesting that dioxygen is not excluded from binding, whereas cyanide, a ligand that forms a linear complex, is sterically excluded by the 10-methyl of (17).

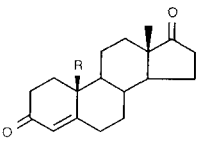
Industrial interest in 10- and 19-substituted analogues has been maintained by Merrell-Dow and some of the first mechanism-based (category (c)) substrate analogues were developed by the Cincinnati-based group.

Hopeful of designing an alkylating agent towards the haem-site of AR, 17β-hydroxy-10-methylthioestra-1,4-dien-3-one (79) was synthesized and had better affinity (apparent K_i = 45 nM) for the binding site than testosterone (K_m = 610 nM). The inhibition by this compound was not entirely enzyme-activated, as it was irreversibly deactivated in the absence of NADPH, although interestingly it was shown that the half-life for AR was decreased when NADPH was included in the incubation medium [207].

10β-Substituted propargyl substrate analogues were synthesized by design by Johnston, Wright and Metcalf [208] as compounds with potential to irreversibly bind to the active site. These workers reasoned that an acetylene-containing substituent may be metabolized by AR to generate a conjugated ketone. The reactive ketone would be an effective Michael acceptor and may well be attacked by an active site nucleophile leading to covalent modification of the enzyme.



(79) R= SMe



(80) R= $CH_2C{\equiv}CH$

(81) R= 1-oxo-2-propynyl

(82) R= 1-hydroxy-2-(trimethylsilyl)ethyl

(83) R= $CH_2CH_2Si\,Me_3$

The 10β-propargylestr-4-ene-3,17-dione (80), 10β-[(1*S*)-1-hydroxy-2-propynyl]estr-4-ene-3,17-dione (73),10β-[(1*R*)-1-hydroxy-2-propynyl]estr-4-ene-3,17-dione (72) and 10β-[1-oxo-2-propynyl]estr-4-ene-3,17-dione (81) were tested against AR, leading to activities shown in *Table 2.1.* Interesting results were obtained for this series of derivatives: compounds (80) and (73) were both found to be enzyme-activated irreversible inhibitors of AR. The diastereoisomeric alcohol (72) acted as a pure reversible competitive inhibitor. The 10β-[1-oxo-2-propynyl] derivative (81), being an inherently reactive

Table 2.1. 10β-PROPYNYL-SUBSTITUTED ANDROSTENEDIONE ANALOGUES AND THEIR INHIBITORY ACTION AGAINST AROMATASE [204]

Derivative	K_i[a] *(nM)*	K_m[b] *(nM)*	K_i/K_m	k_{inact} ($\times 10^{-3}\ s^{-1}$)
(80)	68	129	0.52	1.11
(73)	21000	50	420	2.91
(72)	2500	60	42	–
(81)	830	41	20	0.54

[a] Apparent inhibition.
[b] Androstenedione substrate.

ketone, inhibited the enzyme irreversibly without metabolic activation, that is, in the absence of NADPH, but, surprisingly the $t_{1/2}$ increased: 21.3 min as compared with 3.6 min [204]. It has been suggested the the deactivation of AR by these derivatives proceeds through a highly reactive oxirene species generated insertion of oxygen by the enzyme into the acetylenic bond [209]; this would readily explain the enhanced activity of (81) in the screening experiment above.

Further to this work was the synthesis of the Δ-6 analogues of 10β-[(1*S*)- (73) and 10β-[(1*R*)- (72) 1-hydroxy-2-propynyl]estr-4-ene-3,17-diones by the same group. In contrast to their reduced analogues, both enantiomers exhibited enzyme activated time-dependent irreversible deactivation of AR (see above) [154].

A novel silylated 10-substituted analogue of androstenedione was synthesized as part of a programme to develop an enzyme-activated irreversible inhibitor of AR. 10β-[1-Hydroxy-2-(trimethylsilyl)ethyl]estr-4-ene-3,17-dione (82) was the only analogue of four substrate-based silylated inhibitors that exhibited any activity. This derivative (82) showed good affinity for the enzyme ($K_i = 562 \pm 12$ nM) but marginal irreversibility. The 19-deoxy analogue (83) did not inhibit AR, which suggests that the hydroxy group in (82) confers an ability for this analogue to approach a binding site (*cf.* 3-methylene analogues) [164].

An extensive investigation of the scope of inhibition by 10β-substituted androstenediones was reported. The compounds shown in *Table 2.2* were all evaluated both as reversible and irreversible inhibitors of AR. The reader is recommended to consult this paper for the synthetic details of these important compounds [168].

10β-Aminoestr-4-ene-3,17-dione (84) was synthesized and found to have low inhibitory activity [210], the corresponding 10β-hydroxyestr-4-ene-3,17-dione (85) has comparable low competitive reversible action [211]. Derivatization of the 10β-amino compound to the corresponding methyl carbamate (86) and isocyanate (87) lowered the activity further. An attempt to synthesize the homologous 19-amino-4-androstene-3,17-dione (88) failed, although the trifluoroacetyl- (89) and acetyl- (90) amino derivatives, and the 19-oximes (91) were made as part of the synthetic route towards (88); all exhibited poor activity. The failure of the 10β-amino compound (84) to act as a potent inhibitor of AR was deemed unexpected by this group as successful inhibition of cholesterol side-chain cleavage enzyme by aza bioisosteres of cholesterol of high potency has been achieved [210].

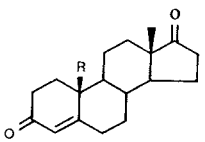
The inactivation of AR by the 10β-hydroperoxy-4-estrene-3,17-dione (92), a steric mimic of the intermediate 19-hydroxy-4-androstene-3,17-dione (19),

Table 2.2. IC_{50} VALUES FOR 10β-SUBSTITUTED DERIVATIVES OF ANDROSTENEDIONE

10-β substituent	*IC_{50} (μM)*[a]
Propargyl	1.0
Allyl	1.8
Propyl	100
Cyanomethyl	2.6
2,2-Difluoroethyl	4.0
2-Fluoroethyl	0.7
2-Chloroethyl	8.0
Ethynyl	1.0
Vinyl	0.1
Ethyl	0.3
Cyano	2.5
Difluoromethyl	4.0
Fluoromethyl	3.0
Chloromethyl	1.4

[a] A substrate concentration of 1 μM androstenedione was used [168].

has been investigated. In the absence of NADPH, the compound showed time-dependent loss of activity of the enzyme. In the presence of NADPH, the compound acted only as a competitive reversible inhibitor, but showing high affinity (K_i = 330 nM, K_m (androstenedione) = 21 nM). Loss of activity of



(84) R= NH_2

(85) R= OH

(86) R= NHCOOMe

(87) R= NCO

(88) R= CH_2NH_2

(89) R= $CH_2NHCOCF_3$

(90) R= $CH_2NHCOMe$

(91) R= CH=NOH

(92) R= OOH

(93) R= CH_2N_3

(94) R= CH_2SMe

(95) R= SSO_2Me

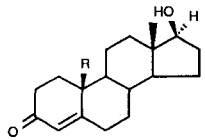
(96) R= CH_2SH

(97) R= oxiranyl

(98) R= thiiranyl

AR was also found to be induced by the nonspecific agents, hydrogen peroxide and cumene hydroperoxide, but at considerably higher concentrations [211].

The 19-azido (93) and 19-methylthio (94) 4-androstene-3,17-diones have been found to be potent competitive reversible inhibitors (K_i = 5 nM and K_i = 1 nM respectively, for K_m (androstenedione) = 25 nM). The same workers also discovered that 19-methanesulphonylthioandrostene-3,17-dione (95) inactivates AR in the presence of NADPH and O_2, although no kinetic or inhibitory data were presented [213]. The interaction of (94) with the active site was found to differ from that of (95) in that the latter displaces the substrate steroid from its binding site and on metabolism deactivates the enzyme, whereas (94) interacts with the substrate binding site but also with the haem-iron complex via a postulated coordinate bond. The differences in binding were deduced by examination of the ultraviolet spectral changes induced by the addition of the two inhibitors to the AR enzyme preparation.



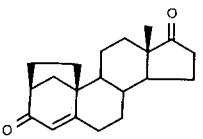
(99) R= SH

A group designing possible suicide substrates of AR have synthesized 17β-hydroxy-10β-mercaptoestr-4-ene-3-one (99) and 19-mercaptoandrost-4-ene-3,17-dione (96) [212]. At variance with the 10β-amino (84) and 10β-hydroxy (85) estr-4-ene-3,17-dione analogues, the 17β-hydroxy-10β-mercaptoestr-4-en-3-one (99) was found to be enzyme-activated, inhibiting AR in a time-dependent manner (apparent K_i = 106 nM, $k_{inact} = 3.2 \times 10^{-3}\ s^{-1}$) and demonstrates high affinity for the enzyme. The 19-mercapto compound (96) is also a potent suicide substrate of AR (apparent K_i = 34 nM, $k_{inact} = 1.2 \times 10^{-3}\ s^{-1}$). The paper gives no data on the interaction of these compounds with the active site as the previous workers did, and it would be of interest to obtain their ultraviolet spectral data. The generation of a sulphenic acid was suggested as the possible reactive intermediate involved in the irreversible binding of (96) and (99) to AR.

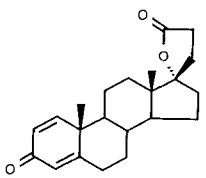
The 10-oxiranyl- (97) and 10-thiiranyl- (98) estr-4-ene-3,17-diones have recently been synthesized and screened for both time-dependent and reversible activity against AR [214]. It was found that their diastereoisomers exhibited only competitive reversible inhibition of the enzyme but with stereoselec-

tivity. The 19*R*-isomers were potent inhibitors, the oxiranyl species was *ca.* 36-times more potent than its enantiomer and the thiiranyl *ca.* 80-fold. The inhibition constants for the (19*R*)-10-oxirane (K_i = 10 nM) and the (19*R*)-10-thiirane (K_i = 2 nM) indicate a greater binding affinity than for either androgen substrate (K_m (androstenedione) = 130 nM). The ultraviolet spectral shifts induced in the AR enzyme preparation by the 19*R*-isomers of both systems suggest that they both interact not only with the substrate binding site but also with the haem-iron complex. Further data obtained by X-ray analysis of the (19*R*)- and (19*S*)-10-oxiranyl derivatives demonstrate that the hetero atom of the three membered ring of the former is positioned over the A-ring directed towards 1- and 2, whereas in the latter it is directed towards the B-ring at position 6 of the steroid nucleus. It was suggested that if the solid-state conformation of the 19*R* compounds represents the conformation of the steroid in the active site, the location of the haem-moiety would be above the 1 and 2 positions as well as 19 and that this would seem to re-enforce the suggestion that 1 and 2 are possible sites of the third hydroxylation in the aromatization cycle. The interaction with the haem-iron complex was considered to be via direct hetero atom lone-pair–iron bonding. These compounds fall into the category (b) rather than (c) since there was no demonstrable irreversibility.

Recent interest has been shown by SKF in the bio-activity of an analogue of androstenedione (17). A derivative of (17) was synthesized with an ethano bridge between the axial 10 and 2 positions (100). This bridge was demonstrated, by X-ray analysis, to alter considerably the conformation of the steroid A-ring and the 19-carbon leaned towards the centre of the A-ring as compared with (17). The biological data for this compound are to be reported in the near future [215].



(100)



(101)

MISCELLANEOUS STEROIDAL INHIBITORS OF AR

It has been suggested that 5α-androstane-3,17-dione, which binds to AR with a similar affinity to that of testosterone, is a natural regulatory inhibitor in the

physiological control of oestrogen biosynthesis [216]. No report of any other derivatives with a reduced 3,4-double bond has been recorded.

A D-ring lactone steroid, Δ^1-testololactone (101), is used in the clinic in the treatment of breast cancer [217] and has also been described as a competitive inhibitor for AR [216]. More recent findings have shown (101) to be a suicide inhibitor which inactivates AR in a NADPH-dependent process with an apparent K_m of 350 nM [218]. However, testolactone itself which lacks the Δ^{-1}-double bond is a simple competitive inhibitor [218]. This finding is consistent with the data described for the Δ^1-derivatives of androstenedione (see p. 273) and seems to reinforce the suggestion that the irreversible action of this type of derivative is due to activation of the Δ^1-double bond or to the lack of an 'extractable' 1β-hydrogen.

CONCLUSIONS

Non-steroidal and steroidal inhibitors of high potency against aromatase have been developed over the past few years. The rational design of compounds, utilizing proposed mechanistic aspects of the enzyme, has resulted in both reversible and irreversible inhibitors capable of inactivating the target enzyme.

Most of the inhibitors covered above have been synthesized as a result of academic interest in the mechanism of the enzyme and only recently have medicinal chemists applied themselves to developing compounds of therapeutic use. As yet only two drugs, aminoglutethimide and 4-hydroxyandrostenedione, are currently used in the clinic against this potential target, both of which have serious failings. Therefore, the need is still strong for compounds that will help map the active site and elucidate the mechanism of action of the enzyme and thereby lead on, hopefully, to a range of potential drugs for the clinician to choose from to treat oestrogen-dependent breast cancer.

ACKNOWLEDGEMENTS

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3 Endocrine Treatment of Prostate Cancer

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INTRODUCTION

The prostate gland is one of the most interesting but unusual organs in the human body. This relatively inaccessible gland (*Figure 3.1*) is not essential for life and its real function is unknown, although it seems reasonable to suppose that it is concerned in the process of maintaining sperm viability. The gland is of particular interest to the urologist and endocrinologist, however, since it has the propensity to cause trouble later in life from inflammatory disorders during the middle-age period and from tumour development, benign adenomas or malignant carcinoma in the older man. A large proportion of males over the age of 40 will develop benign prostatic hyperplasia and in England and Wales, carcinoma of the prostate is the fourth most common cause of death from malignant disease, being responsible for approximately 4,000

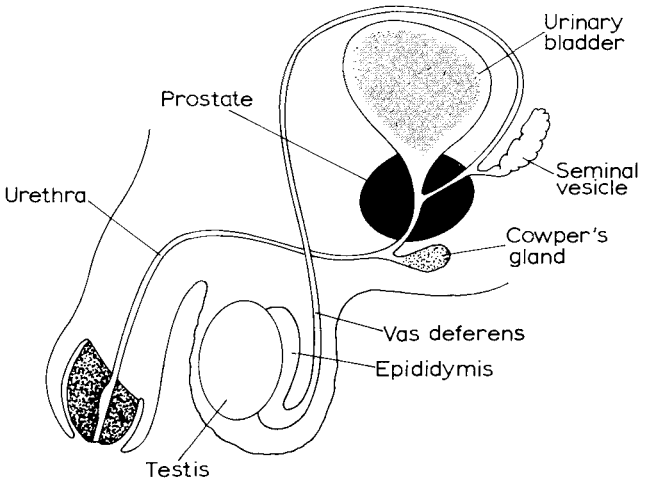


Figure 3.1. Prostate gland and the surrounding organs in man.

deaths each year [1]. In the United States of America, prostatic cancer is the second most common form of malignancy in men [2, 3] with over 50,000 new cases and 18,000 deaths annually [4]. Carcinoma of the prostate is essentially a disease associated with advancing years, being diagnosed only occasionally under the age of 50, and is often present, but asymptomatic, in older men dying from other causes. Carcinoma of the prostate is indeed a common disease. Critical histological analysis of tissue resected for the treatment of benign prostatic hyperplasia had demonstrated an increasing incidence of carcinoma with age; approximately 30% of men in their 50's and 90% of men aged 90 were reported with 'incidental' carcinoma after pathological examination. The factors that are concerned with the promotion of these small asymptomatic areas of cancer into the aggressive malignant disease remain unknown [5, 6], but clearly the role of the androgenic steroids in such a process is probably of paramount importance and illustrates the reason for the particular interest of the endocrinologist in prostatic carcinoma.

SCIENTIFIC BASIS FOR THE ENDOCRINE TREATMENT OF PROSTATIC CANCER

The normal prostate is dependent upon the testicular synthesis and secretion of testosterone to maintain cellular integrity and functional activity of the

gland. It has been known from earliest times that castration prevents the normal development of the secondary sexual characteristics, although it is generally accepted that our understanding of the testicular control of the accessory sex glands, including the prostate, began with the report of Hunter in 1786 [7]. References to the effectiveness of orchidectomy for the treatment of the enlarged prostate appeared, however, only in the late 1800's [8, 9].

Hampton Young [10] described the first total prostatectomy, performed in Johns Hopkins Hospital, U.S.A., for the treatment of prostatic cancer and discussion remains as to the value of this operation, certainly in the management of early disease confined to the gland, when impotence and often incontinence are noted subsequent side-effects of such treatment.

The scientific foundation on which the endocrine treatment for the management of advanced prostatic cancer, when the disease has spread beyond the capsule of the gland, is based, was established from studies in the late 1930's. Investigations [11, 12] had indicated both that metastatic prostate cancer cells retained the capacity to produce acid phosphatase, a characteristic of the normal prostate cell, and that the concentration of this enzyme was related to testicular activity [13]. Huggins and Hodges [14] then proposed that prostatic cancer might retain some degree of responsiveness to androgen control as known with the normal prostate gland. By introducing the concept of 'anti-androgen therapy', orchidectomy or the use of the newly synthesized orally active oestrogen, diethylstilboestrol (DES, (1), see p. 304) Huggins' team showed [15] that patients with prostatic cancer, with painful metastatic deposits in bone and consequently elevated plasma acid phosphatase levels, clinically responded well and enzyme levels in blood were reduced [16, 17].

Since that time, DES has generally been accepted as an inexpensive but effective oestrogen for the management of advanced prostatic cancer and this, or orchidectomy, became the preferred choice for first-line therapy. Most of the accumulated evidence indicates that DES exercises its anti-androgenic role by decreasing luteinizing hormone (LH) synthesis and secretion by the pituitary gland (*Figure 3.2*), although it has also been shown to have a direct action on both the testis and the prostate gland [5].

Schematically illustrated in *Figure 3.2* are the inter-relationships between the glands and hormones that are associated with the regulation of prostatic growth and function. The biological process concerned with the transfer of releasing factors from the hypothalamus to the pituitary with the resultant release of pituitary hormones is currently a topic of considerable interest. The LH secreted by the pituitary consequently controls the synthesis and secretion of testosterone by the testes. At the target organ, the prostate, testosterone is converted to 5α-dihydrotestosterone, considered to be the active

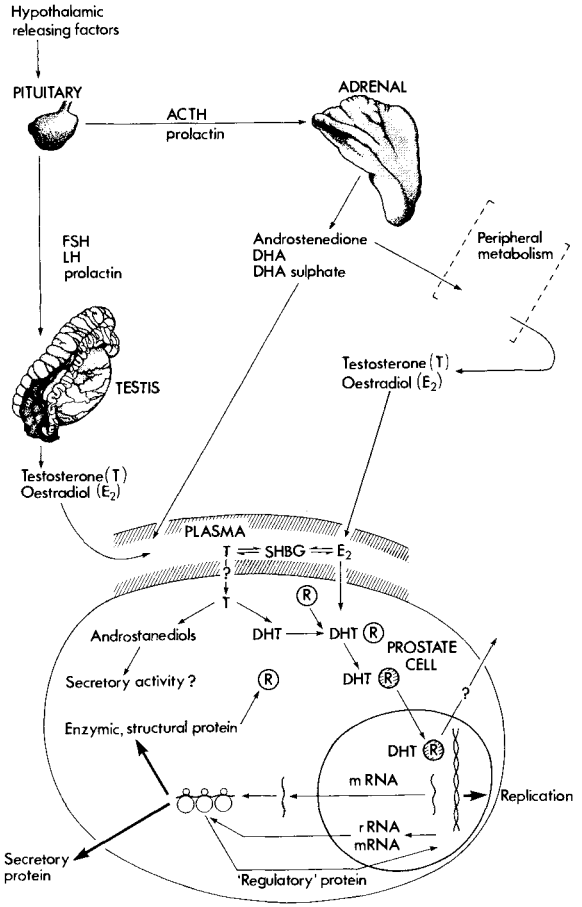


Figure 3.2. Schematic illustration of the hormonal control mechanisms of the prostate cell.

androgenic steroid, which then associates with the steroid-receptor protein to form a complex. This complex binds to acceptor sites on the nuclear chromatin and controls the various biochemical processes concerned with gene transcription and, ultimately, protein synthesis [18].

Data from many years of experimental animal research have tended to suggest that the steroids produced by the adrenal gland may also influence the growth and function of the prostate gland [19–22], and there is at present considerable interest in the role of the adrenal androgens in promoting the growth of human prostatic cancer [6, 23, 24]. These adrenal C_{19}-steroids,

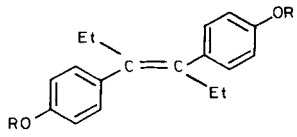
dehydroepiandrosterone (DHA) sulphate, DHA and androstenedione are certainly metabolized, peripherally, to testosterone and, furthermore, are converted within the prostate gland to testosterone and 5α-dihydrotestosterone, although not very actively (*Figure 3.2*), [25, 26]. Moreover, the evidence that the adrenal steroids have any real biological effect on the prostate gland of the rat must be considered somewhat equivocal. Adrenalectomy has little effect on the size of the prostate gland and after orchidectomy the prostate gland together with the other accessory sex organs quickly atrophy. Furthermore, in man, no compensatory increase in adrenal C_{19}-steroid synthesis occurs after orchidectomy, [27, 28] although the precise role of the 'residual' serum adrenal androgens, in relation to the progression of prostatic cancer may still require more careful consideration. The concentration of testosterone in serum falls to approximately 2 nmol/l, the 'castrate' level, after orchidectomy, and there is adequate evidence to indicate that this 'residual' testosterone is derived from adrenal precursors [29, 30]. Since the early 1940's, therefore, following the pioneering work of Huggins and his colleagues, the basis of the first-line treatment of advanced prostatic cancer was to lower the serum testosterone concentration by either bilateral orchidectomy or administration of DES. It is generally accepted that approximately 70–75% of patients will initially respond to this form of endocrine therapy [31], although it is well recognized that the treatment is only palliative [32]. Relapse, which is rarely associated with elevated serum levels of androgen [33], usually occurs within 2 years [34, 35] and the median survival is then 6 months. It is generally accepted that relapse is merely an expression of the inexorable progression of the growth of androgen-independent clones of cancer cells probably present from the outset in the primary tumour.

OTHER FORMS OF ENDOCRINE THERAPY

The basis of endocrine therapy for the management of prostatic cancer is therefore withdrawal of testosterone by castration, the suppression of testicular activity with oestrogens or the blockade of the androgenic action at the level of the prostate cell by anti-androgens.

The principal therapeutic effect of the oestrogens is considered to be on the pituitary, inhibiting LH secretion, thereby suppressing the synthesis of testosterone by the testis. DES has generally been the most acceptable drug for the management of advanced prostate cancer, although concern was expressed following the Veterans Administration Cooperative Urological Research Group's (VACURG) report on the associated side-effects, which

include thromboembolic disease, impotence, gynaecomastia, genital atrophy and nausea [36–38]. These side-effects are dose-related, however, and are minimal when 1 mg DES is administered daily. For the effective management of the disease, it is generally considered necessary to give 1 mg DES, t.d.s. although even this dosage may not completely suppress testicular activity [39].



(1) R = H
(2) R = OPO_3H_2

There has long been the belief that DES may have a direct local action on the prostatic cancer, and it has been claimed [40] that orchidectomy and DES was clinically more effective than either form of therapy alone. The rationale behind the use of fosfestrol (DES 4,4′-diphosphoric acid ester, Honvan, (2)) in large doses (200 mg daily) is that the free and locally active form of the drug DES would be released within the tissue by the high concentrations of phosphatases present in prostatic cells. There is little evidence that fosfestrol accumulates in the prostate or that DES is released in reasonable concentrations [41]. Such evidence, however, still does not preclude the possibility that DES has a cytotoxic action in the prostatic cell and studies continue to attempt to identify this effect.

Other oestrogens which have been used to treat prostatic cancer have failed to receive universal acceptance. Polyoestradiol phosphate (Estradurin), a long-acting oestrogenic preparation given intramuscularly, is well used in Scandinavia but only weakly suppresses pituitary activity [42] and neither Premarin, a mixture of conjugated equine oestrogens (2.5 mg, t.d.s.), nor ethynyloestradiol (0.15–1.0 mg/day), has been universally accepted.

Various progestational steroids such as chlormadinone acetate, medroxyprogesterone acetate (Provera), hydroxyprogesterone caproate (Delalutin) and megestrol acetate (Megace) have been used to manage the patient with carcinoma of the prostate. Such compounds inhibit LH release from the pituitary and have been reported to be weakly anti-androgenic within the prostate. Reasonably good clinical results from the use of progestational steroids have been described [43], but the real value of these drugs has never been assessed by controlled and randomized clinical trials.

Cyproterone acetate (Androcur), a weak progestational steroid, also has an

anti-androgenic effect, inhibiting the binding of androgen to receptor protein in the prostate [44]. Although an apparent alternative to DES therapy [45], and despite extensive clinical investigation, [43, 46–48], its precise role for the treatment of prostatic cancer still remains to be established [49].

Flutamide, a non-steroidal anti-androgen, has no progestational effects and therefore does not suppress plasma levels of testosterone. Its administration results in an increased secretion of LH through inhibition of the feed-back control of the hypothalamic-pituitary axis by androgens and its clinical use alone has rarely been contemplated.

Orchidectomy, the operation to remove the major source of testosterone, is still seen, nearly 50 years after the early experimental work of Charles Huggins, as the basic, simplest form of therapy for prostatic cancer. The use of DES now causes some degree of concern [38] and other drugs have never

Table 3.1. PLASMA TESTOSTERONE

Plasma testosterone concentrations of patients with prostatic cancer who have undergone surgery and of non-castrated elderly men before (–) and after (+) stimulation using human chorionic gonadotrophin (3000 IU).

	Testosterone (nmol/l)	
	–	+
Total orchidectomy group	0.42	0.35
	1.30	0.73
	0.63	0.73
	0.73	0.59
	0.66	0.55
	1.25	1.18
Subcapsular orchidectomy group	1.56	0.83
	0.64	0.49
	0.99	1.04
	1.16	1.70
	0.71	0.66
	0.62	0.76
	1.28	1.98
Non-castrated elderly men[a]	10.1	22.4
	9.9	17.9
	10.8	17.1
	11.4	14.3
	15.2	27.9

[a] Wilcoxon matched-pairs signed-rank.

been found to be generally acceptable. In the United Kingdom, in particular, there has been a reversion from the use of DES to subcapsular orchidectomy as the first-line endocrine treatment of choice. First introduced in the early 1940's [50], its clinical use was challenged by McDonald and Calams [51], who reported that a proportion of Leydig cells remained on the tunica albuginea after the operation. Information from various centres [52, 53], including our own (*Table 3.1*), has subsequently demonstrated that the operation effectively removes all Leydig tissue and the surgical result is aesthetically acceptable to the patient. Clearly, little progress would seem to have been made since the early 1940's and the initiative resulting from exciting new experimental work with specifically synthesized analogues of luteinizing hormone-releasing hormone (LH-RH) has been eagerly accepted by those clinically concerned in the management of carcinoma of the prostate.

LUTEINIZING HORMONE-RELEASING HORMONE AND ITS ANALOGUES

It is more than 30 years since Harris [54] proposed that pituitary function was under the neural control of the hypothalamus and that the secretion of pituitary hormone was governed by releasing factors originating in the hypothalamus. Since the early reports [55, 56] that hypothalamic extracts caused the release of LH from the pituitary gland, enormous progress has been made in the isolation and characterization of the hypothalamic peptide, LH-RH. Matsuo, Baba, Nair, Arimura and Schally [57, 58] isolated LH-RH from porcine hypothalami and Amoss, Burgus, Blackwell, Vale, Fellows and Guillemin [59] obtained it from ovine tissue. The significance of these achievements was later recognized in 1978 by the award of the Nobel Prize for Medicine to Schally and Guillemin, the heads of these two research teams.

Naturally occurring LH-RH is a decapeptide (*Figure 3.3*) produced by the

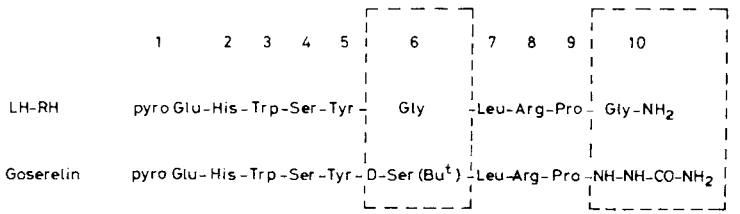


Figure 3.3. The structure of human luteinizing hormone-releasing hormone and its analogue goserelin.

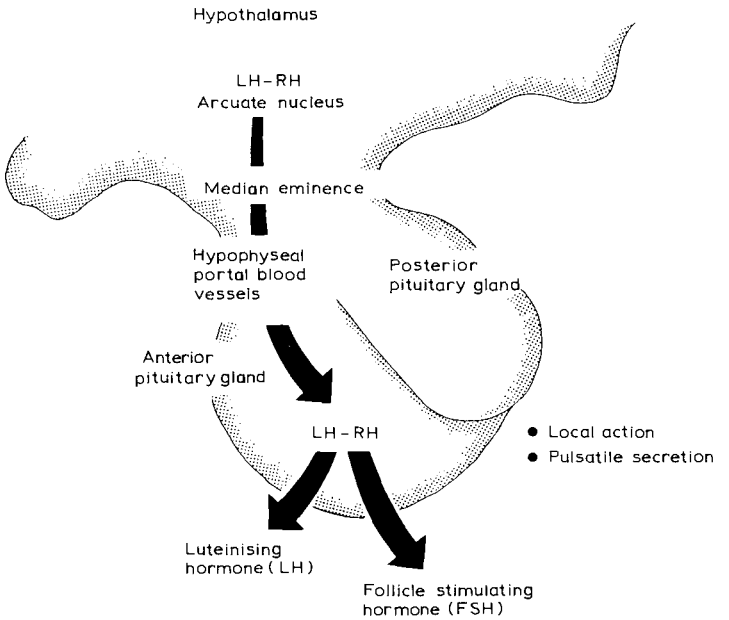


Figure 3.4. Production of luteinizing hormone-releasing hormone from hypothalamus and its control of pituitary peptide hormone secretion.

median, pre-optic and suprachiasmatic areas of the hypothalamus [60] (*Figure 3.4*). Immunohistochemical localization has also demonstrated that the peptide can be found in the median eminence and the arcuate nucleus [61]. Further study [62, 63] indicated that LR-RH was also localized in extrahypothalamic areas of the brain, and it was suggested that the peptide may act as a neurotransmitter.

LH-RH is released from the hypothalamus in a pulsatile manner, and carried along the neurons originating in the arcuate nucleus to the hypothalamopituitary portal system. In the pituitary, it promotes the release of both LH and follicle-stimulating hormone (FSH), although it has been suggested [64] that the pulsatile nature of the LH-RH presentation to the pituitary regulates LH secretion, whereas the more prolonged exposure of the gland to LH-RH results in FSH release.

Since the isolation of LH-RH, and the confirmation of its amino-acid sequence [65], many LH-RH analogues have been synthesized in the search for molecules with potential in clinical medicine. Many have been reported to have pronounced physiological and pharmacological effects [66–68]. Acute

administration of these analogues stimulated the secretion of gonadotrophins from the pituitary with consequent increased synthesis of testosterone by the testes [69, 70]. Long-term administration of pharmacological doses of LH-RH analogues paradoxically produced an antigonadal effect in both male and female rats. In male rats, they decreased LH and prolactin receptors in the testis, decreased serum concentration of testosterone, arrested spermatogenesis and reduced the weight of the testis, prostate gland and the accessory sex organs [71–74].

Studies at the Tenovus Institute were concerned with the potent LH-RH analogue, goserelin (ICI 118630 (Zoladex), CAS Registry No. 65807-02-5, *Figure 3.3*), with an *O-t*-butyl-D-serine at position 6 and an azaglycinamide residue ($NHNHCONH_2$) at position 10 [75]. Administration of a single injection (5 μg) of goserelin to intact male rats increased the serum levels of FSH and LH, with concentrations peaking after 2 h and returning to normal base-line levels within 12 h. Injection of 5 μg of goserelin twice daily for 10 days significantly decreased the concentration of serum testosterone and the weights of the testes and accessory sex glands. Experimental 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced mammary tumours of female rats were also shown to regress after administration of goserelin [79] and other similar analogues [77, 78].

Such experimental results, and the observation that LH-RH analogue administration inhibited hormone-dependent prostatic tumour growth in animals [79], directed attention to the potential therapeutic effect of these agonists for the treatment of patients with advanced carcinoma of the prostate and for the clinical management of premenopausal women with cancer of the breast. This exciting advance has provided the clinician with a new initiative in the field of prostatic cancer treatment.

PHASE I AND PHASE II CLINICAL STUDIES WITH GOSERELIN

Early clinical studies established between the Institute and the Yorkshire Prostatic Cancer Group [80] showed that daily subcutaneous administration of goserelin (Zoladex) to patients with histologically proven carcinoma of the prostate, with or without metastatic disease, resulted in a decrease in the concentration of testosterone in serum to castrate levels. Four patients received 250 μg/day for periods up to 3 months and four received 100 μg daily. In the four patients on the higher dose, the plasma testosterone concentration reached this level by day 14, whereas on the daily 100 μg dose, castrate levels were achieved after 5–6 weeks (*Figure 3.5*). Comparable changes were observed in the serum levels of FSH and LH, and were associated with a

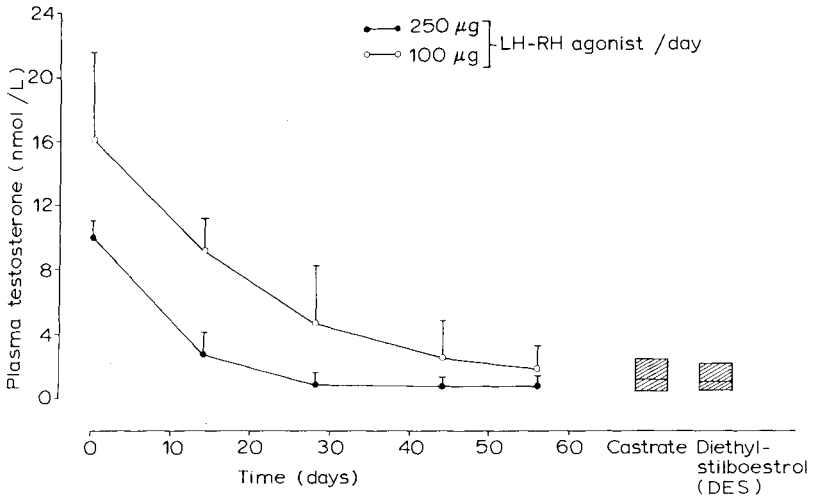


Figure 3.5. Changes in plasma testosterone concentrations in advanced prostatic cancer patients following daily injections of goserelin.

significant clinical improvement in 7/8 patients, without any relevant side-effects.

The more recent development of a slow-release (depot) formulation of goserelin [81] has allowed the administration of the agonist as a single monthly injection. Such a depot preparation has obvious clinical advantages. The peptide was incorporated in a 50:50 lactide-glycolide copolymer in the form of a small cylindrical rod, which can be injected, under local anaesthesia, through a 16-gauge needle, into the subcutaneous tissue of the anterior abdominal wall.

The effectiveness of the depot preparation was assessed in 22 patients with carcinoma of the prostate with metastatic disease [81]. Patients were given, at random, one of three doses of depot goserelin 3.6, 1.8 or 0.9 mg every 28 days, doses corresponding to the average release of the LH-RH agonist at a rate of 120, 60 or 30 μg/day, respectively, for the 28 days.

Within 1 h of the subcutaneous injection of the depot formulation, an increase was observed in serum concentrations of LH and FSH in patients on each of the dose levels of the agonist. The concentration of serum LH increased during the first 4 h before reaching a plateau (*Figure 3.6*) and no clear dose-response relationship was observed. By monitoring hormone levels during the treatment period, it was shown that the LH and FSH concen-

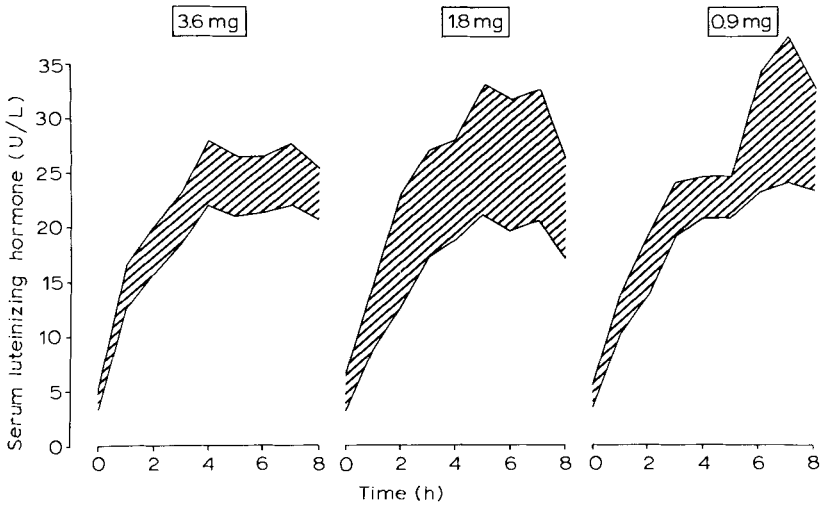


Figure 3.6. Serum luteinizing hormone (LH) concentrations (mean ± S.E.) in patients with advanced prostatic cancer following the primary injection of depot goserelin in three doses (0.9, 1.8 and 3.6 mg/28 days).

trations fell to undetectable levels by day 15 (*Figures 3.7 and 3.8*). Additional injections on days 29 and 57 elicited no further increase in the concentration of LH in these patients (*Figure 3.9*).

The concentration of serum testosterone was unaffected by the administration of the depot during the first 8 h of treatment, but then increased between days 1 and 4, after which, a gradual decrease was observed (*Figure 3.10*), until castrate levels (< 2 nmol/l) were reached between days 15 and 22 in patients on the two higher doses.

This preliminary study clearly indicated that the slow-release depot formulation was effective in producing a medical castration and offered a novel, practical and clinically valuable form by which the LH-RH analogue could be administered. The hormonal changes were associated with subjective clinical improvement, with patients experiencing relief of bone pain. Elevated serum total and acid phosphatase values decreased during treatment, no toxicity was reported and side-effects were minimal, although hot flushes were experienced by some patients as a direct result of androgen withdrawal.

There is an increasing interest in the use of these LH-RH analogues for the treatment of hormone-dependent cancers [67, 82–84]. These preparations, however, were given subcutaneously or intranasally [85–87]. The latter, although relatively simple, may require three doses each day and it is reported

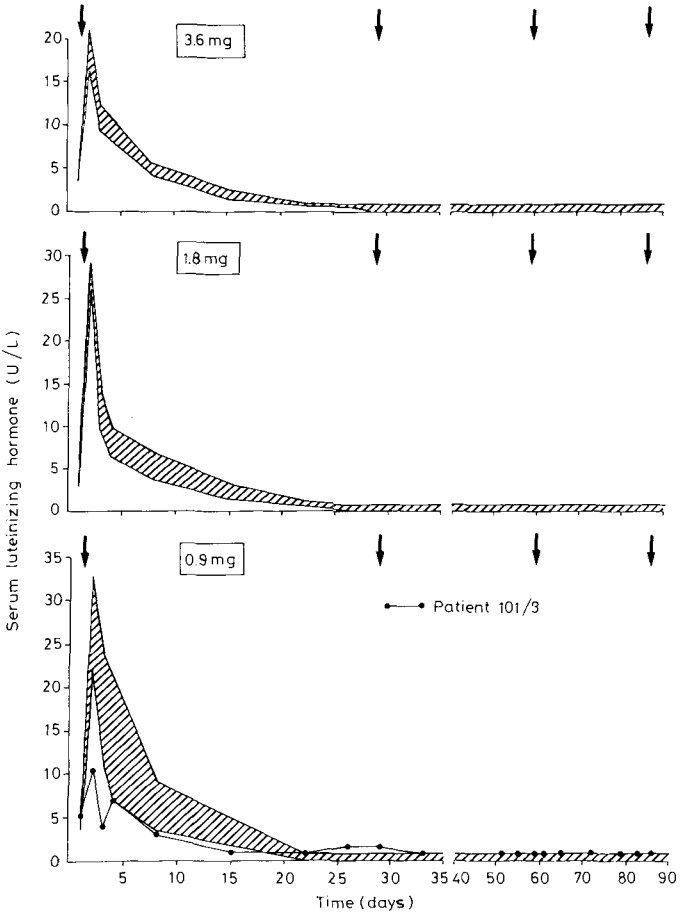


Figure 3.7. Serum luteinizing hormone (LH) concentrations (mean ± S.E.) in patients with advanced prostatic cancer treated with depot goserelin over a 90-day period. LH levels of patient 101/3 shows insufficient suppression at the dose level of 0.9 mg/28 days. Arrows (↓) indicate days of injection.

that only 5% of the analogue is absorbed [67]. The goserelin depot preparation is immediately active, provides a sustained release of the analogue and results in desensitization of the pituitary gland to LH-RH within 15 days.

PHASE III TRIAL: GOSERELIN AGAINST CASTRATION

With increasing concern expressed about the clinical use of DES following the VACURG studies [36, 37], and understanding the physical and psychological

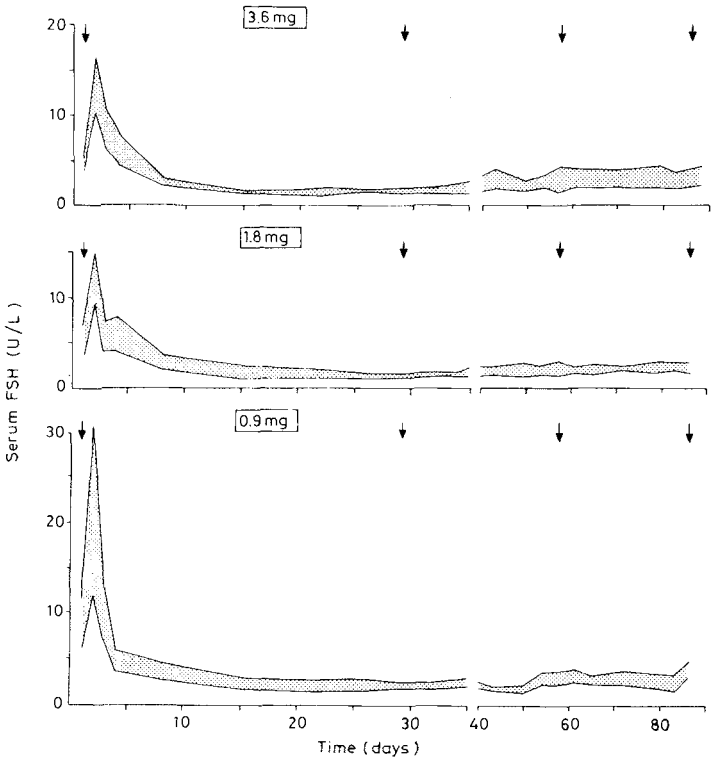


Figure 3.8. Serum follicle stimulating hormone (FSH) concentrations (mean ± S.E.) in patients with advanced prostatic cancer treated with depot goserelin over a 90-day priod. Arrows (↓) indicate days of injection.

stress associated with orchidectomy, there has been, in recent years, a tendency in the United Kingdom to revert to the use of subcapsular orchidectomy for the first-line treatment of metastatic prostatic cancer. This operation is considered cosmetically and psychologically more acceptable, although it is important to bear in mind some of the earlier studies at the Tenovus Institute [88, 89], which drew attention to the 'direct pathway' between epididymis and the prostate via the vas deferens and differential vein where androgens, synthesized by the epididymis from adrenal C_{19}-steroids, could be directly transferred to the prostate. The epididymides are conserved when a patient undergoes subcapsular orchidectomy.

There was, however, complete agreement in the British Prostate Study Group that in the establishment of a randomized clinical trial to consider the

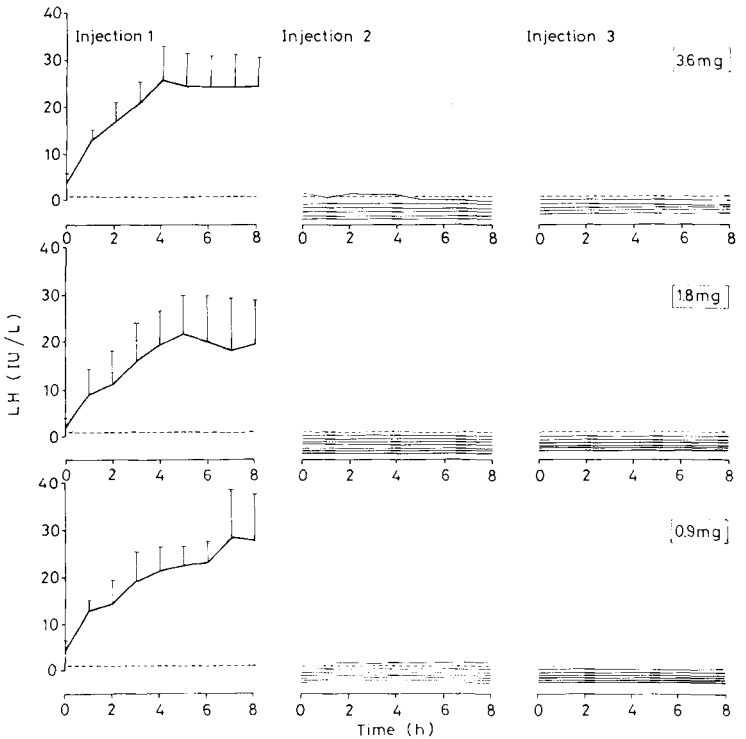


Figure 3.9. Serum luteinizing hormone (LH) concentrations (mean ± S.E.) in patients with advanced prostatic cancer within 8 h following three consecutive injections of depot goserelin in three doses (0.9, 1.8 and 3.6 mg/28 days).

effectiveness of the depot formulation of goserelin, bilateral total or subcapsular orchidectomy were the standard British forms of therapy against which the value of goserelin must be assessed.

An open, randomized phase III clinical trial was conducted in various Urological Centres, primarily in the South-West of the United Kingdom, and co-ordinated by the Tenovus Institute. It was designed to incorporate at least 300 patients with histologically confirmed prostatic cancer, radiological and/or isotopic evidence of bone metastases and/or evidence of soft tissue metastases. A life expectancy of at least 3 months was required and the patients had to be sufficiently fit to undergo orchidectomy [90].

Patients were randomized to receive either 3.6 mg depot goserelin every 28 days or total or subcapsular orchidectomy. After entry, patients were

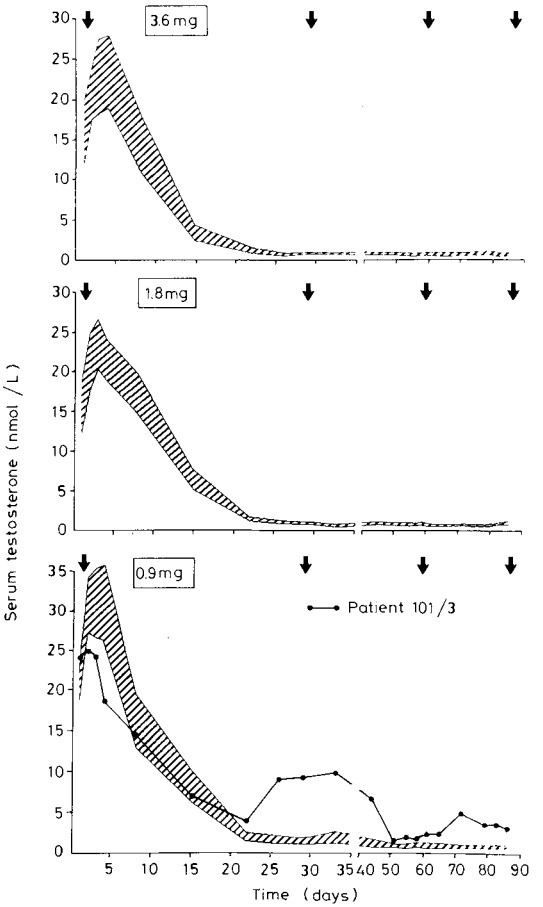


Figure 3.10. Serum testosterone concentrations (mean ± S.E.) in patients with advanced prostatic cancer treated with depot goserelin over a 90-day period. Arrows (↓) indicate days of injection. Testosterone concentrations of patient 101/3 shows inadequate suppression of testicular activity on the lowest dose of 0.9 mg/28 days.

retained in the assigned group until evidence was obtained that there was disease progression, when further treatment was left to the discretion of the various Clinical Centres. All patients were, however, followed to determine survival statistics.

Patients were assessed according to the criteria of the British Prostate Group, which included subjective and objective scoring systems. Criteria for

subjective assessment were based on changes in urine flow, activity scores, bone pain and the use of analgesia. The presence and absence of libido, erections, hot flushes and breast tenderness and swelling were also recorded. Objective assessment was based on the T-category of the primary tumour, prostatic dimensions by either digital examination or prostatic volume, determined by rectal ultrasound, whole body isotope scan and/or X-rays, other clinically measurable metastases and prostatic acid phosphatase in serum.

The trial commenced in October 1984 and the recruitment ceased in February 1986, when 322 patients had been admitted. Preliminary data have been reported [91, 92] on the first 240 patients admitted who had a minimum 3 months documentation. Of the total number of patients, 161 were randomized to orchidectomy and 161 to goserelin therapy. Data on the characteristics of the patients indicated (*Table 3.2*) that there were no significant differences between the treatment groups.

Table 3.2. PHASE III TRIAL: PATIENTS' CHARACTERISTICS
Orchidectomy vs. depot goserelin in the clinical management of patients with advanced carcinoma of the prostate.

	Orchidectomy	*Goserelin*
No. patients entered	161	161
No. protocol violations	17	13
No. eligible for entry	144	148
Age (yr)	73 (55–89)	72 (49–86)
Weight (kg)	71 (46–110)	70 (36–96)

Patients were assessed at 12, 24 and 36 weeks for both objective and subjective responses to treatment. There was no significant difference in objective response rates between the two groups (*Table 3.3*).

Side-effects resulting from both treatments, shown on *Table 3.4*, indicate minimal problems associated with depot goserelin treatment.

Median length of survival was 110 and 91 weeks for patients receiving depot goserelin and those who had undergone orchidectomy, respectively. Estimated survival probability, illustrated in *Figure 3.11*, was also similar for patients on both arms of the treatment. These results clearly indicate that in the hormonal treatment of patients with advanced disease depot goserelin is as effective as the traditional surgical procedures of removal of the testes either

Table 3.3. PHASE III TRIAL: LONG-TERM OBJECTIVE RESPONSE

Orchidectomy vs. depot goserelin in the clinical management of patients with advanced carcinoma of the prostate. Ox, orchidectomy; G, goserelin.

	% response rate					
	12 weeks		*24 weeks*		*36 weeks*	
	Ox(n = 88)	*G(n = 99)*	*Ox(n = 70)*	*G(n = 77)*	*Ox(n = 51)*	*G(n = 59)*
Complete response	0	0	0	0	0	0
Partial response	55	60	45	45	29	29
Stable disease	19	19	8	13	4	8
Disease progression	26	21	42	67	67	63

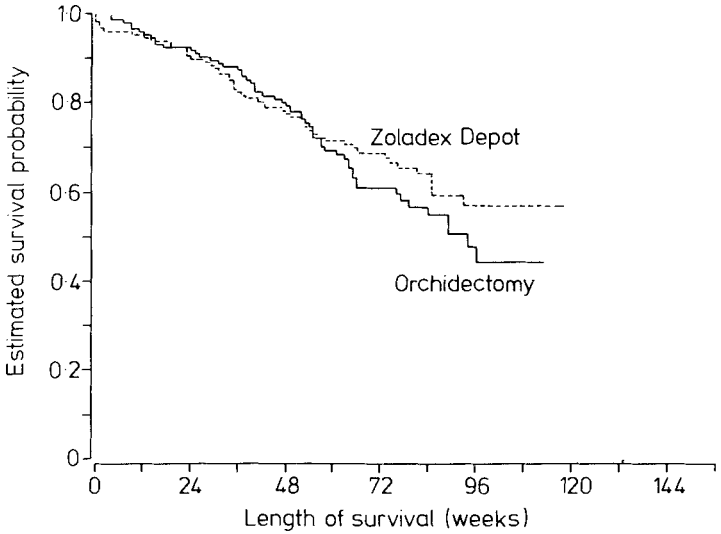


Figure 3.11. Phase III trial. Comparison of estimated survival probability of advanced prostatic cancer patients treated with depot goserelin and orchidectomy.

Table 3.4. PHASE III TRIAL: REPORTED SIDE-EFFECTS, NEW ILLNESSES, SIGNS AND SYMPTOMS

Orchidectomy vs. depot goserelin in the clinical management of patients with advanced prostatic carcinoma.

	Orchidectomy	*Goserelin*
Post-operative complications	18	n/a
Problems at the injection site	n/a	0
Transient increase in pain	0	6
Hot flushes and/or sweats	5	4
Changes in laboratory indices	2	11
Systems: gastro-intestinal	15	28
cerebrovascular	26	34
central nervous system	14	20
respiratory	18	24
urogenital	6	15
musculoskeletal	42	46
skin	3	7
other	29	31

totally or partially. This particular LH-RH analogue therefore offers a valuable alternative therapy to surgery in patient management. New trials are now underway to assess the clinical, long-term efficacy of 'combined' therapy, depot goserelin together with flutamide or cyproterone acetate (Cyprostat) as the anti-androgens against depot goserelin alone for the treatment of advanced, metastatic carcinoma of the prostate.

CONCLUDING REMARKS

These well-controlled clinical trials, although providing invaluable information for the endocrine treatment of the disease, are unlikely to indicate a 'cure' for prostate cancer, since it is well known that hormonal manipulation only provides a transient regression of the cancer. There is also the knowledge that, following progression of the disease, median survival time remains to be approximately 6 months, despite various forms of further hormonal treatment, indicating that the cancer loses hormone dependency. Recent advances that have been made in the field of growth-promoting and -inhibiting factors in relation to various forms of malignancies may provide the basis for further research towards the discovery of other mechanisms of proliferation control in tumours. The availability of new, less toxic and more specific antitumour agents would also encourage further clinical trials assessing their potential therapeutic value. Such trials are currently met by a degree of scepticism due to the well-known, untoward side-effects of presently available cytotoxics on the elderly population.

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4 The Microcomputer in Biomedical Education

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INTRODUCTION

Computer-assisted learning or CAL (also called computer-assisted instruction or CAI) has been around quite a long time now, but the advent of the inexpensive microcomputer in the past 10 years has given great impetus to the use of such machines in education, both in teaching and in management [2]. In this review, we discuss what we see as the advantages of using microcomputers in education in the general area of biochemistry and we project a little into the future. We make no apology for making little reference to CAI projects going on in other subject areas, many of which have been implemented using mainframe computers with little or no graphics capabilities. We believe that low-cost microcomputers with remarkable graphics capabilities offer new ways of using computers in teaching. We also report on the use of computers in assessment and examination, since they have been used in this context with medical students for many years now (see *Table 4.1*).

Table 4.1. EDUCATIONAL OBJECTIVES OF USING MICROCOMPUTERS IN BIOCHEMISTRY

(1) Supporting traditional teaching methods
The animated and interactive blackboard: easy access to attractive and realistic graphics (graphs, preparation of slides, animated molecular models, statistics packages, spreadsheets): possibility of interaction.

(2) Computer-assisted learning (CAL) or instruction (CAI)
Computer assumes the role of instructor (frequent testing with looping back until a section is understood): self-paced, possibilities for problem solving, use of simulations.

(3) Management of education
Maintenance of student records, updating handouts, producing examination papers, marking examinations, producing individualized responses.

(4) (Micro)computers as tools in biochemistry
Interfacing microcomputers with laboratory equipment (controlling, data collection), literature-searching, data-base searching.

THE MICROCOMPUTER

Microcomputers as we now know them, that is, compact stand-alone computers, came into being in the mid-1970's with the introduction of microprocessor chips. These combined all of the components of a computer's central processor unit (CPU) on a single integrated circuit chip.

All modern microcomputers consist of five sections. These are depicted in *Figure 4.1.*

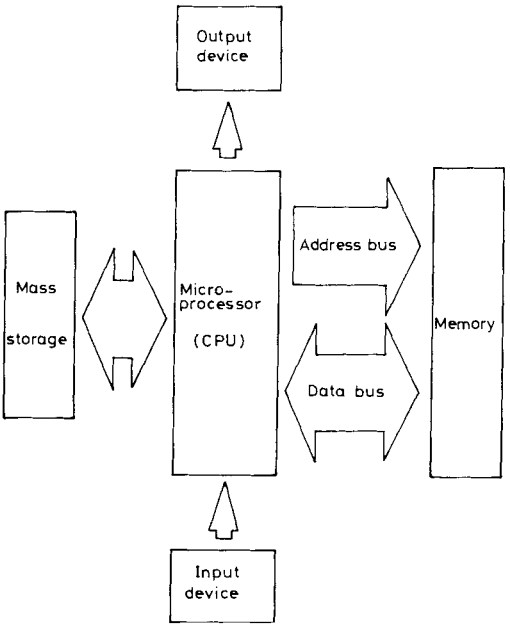


Figure 4.1. Sections of a typical microcomputer.

MEMORY

This is effectively a collection of switches. Each switch can be on or off and can represent a binary digit (abbreviated to 'bit'), where 'on' represents 'one' and 'off' represents 'zero'. The unit of memory is 8 bits, called one 'byte'. There are usually many thousands of bytes in the memory of even the smallest microcomputer and therefore memory size is usually referred to in kilobytes or megabytes. (Since computers use binary arithmetic, 'kilo' actually means 2^{10} or 1024 rather than 10^3. Similarly 'mega' means 2^{20} or 1,048,576 rather than 10^6). Each byte of memory can be regarded as storing a binary number between 00000000 and 11111111, equivalent to 0–255 decimal. These numbers may represent data, programs or even graphics depending on the context decided by the processor.

There are basically two types of memory, called random access memory

(RAM) and read only memory (ROM). While the processor can read the numbers stored in both kinds of memory, it can only alter the contents of RAM. The contents of ROM cannot be altered. The contents of RAM are 'volatile', that is, they are lost when the computer is switched off. This is not the case for ROM and so it is used to hold programs and data that the computer will always need, particularly when it is switched on again.

CPU

This is the heart of the microcomputer. The CPU or microprocessor examines the contents of memory and interprets them as instructions or data. The way in which the processor interprets the numbers stored in memory depends on the design of the chip, that is, on the manufacturer. This is the *machine code* and the microprocessor's repertoire of arithmetic and logic functions is called its *instruction set.* The CPU is connected to the memory by two sets of wires called the *data bus* and the *address bus.* The data bus is used to transfer data to and from the memory. The address bus is used to identify that part of memory with which the processor wishes to communicate. It is important that the processor and the memory act in a synchronized manner. All microcomputers contain a crystal-controlled oscillator which acts like a metronome to which all actions are synchronized. This oscillator is sometimes referred to as the *clock.*

The speed at which a microcomputer can operate will be a function of the frequency of the clock, the time taken for each instruction to be executed (measured in clock cycles), and the number of wires in the data bus. If there are eight wires in the data bus, then the processor will be able to read or write one byte at a time to or from memory. If there are more wires, it can handle more bytes at a time and will therefore be able to operate more quickly. The older microprocessors had clock frequencies of 1 MHz and had 8 wires in the data bus and are referred to as '8-bit' microprocessors. The modern processors such as the Intel 80386 and the Motorola 68020 have clock frequencies of 15–25 MHz and 32 wires in the data bus – so they are '32-bit' processors. One might therefore expect something like an 80-fold increase in speed between the old and new processors. In practice, this is usually partially offset by the fact that the more sophisticated processors have more sophisticated functions which take more clock cycles to execute. However, a few modern processors have greatly simplified instruction sets and these RISC (Reduced Instruction Set Chip) machines are very fast indeed.

The amount of memory a computer can use will depend on the number of wires in the address bus. The older microprocessors generally had 16 wires

in their address buses. They could therefore address 2^{16} or 64 kilobytes of memory and the microcomputers containing these microprocessors were sold as '64K' machines. The IBM-PC contained an Intel 8088 microprocessor. This has a 20-wire address bus capable of handling 1 megabyte of memory. However, the IBM-PC reserves 384 kilobytes of memory for ROM and graphics, so the maximum RAM that can be used directly is 640 kilobytes. At the time, this was not considered to be a great limitation and all of the programs were written for this configuration. The newer IBM PS/2 machines have Intel 80286 and 80386 processors which have 'wider' address buses capable of handling much more memory. However, at the time of writing, existing programs still assume the PC architecture and, although the new machines may have several megabytes of RAM, only 640 kilobytes of it can be used directly!

OUTPUT DEVICE

All microcomputers have at least one output device, usually a monitor screen with a printer as a second output device giving hard copy. On desk-top computers, the screen is usually a cathode ray tube, while on portable computers liquid crystal or gas plasma screens are used. Early screen displays simply emulated the teletypes used at that time to communicate with mainframe computers. These 'glass teletypes' displayed only characters. The computer simply sent a stream of bytes to the output device. The device was electronically connected to the microcomputer in such a way that from the microprocessor's point of view the output device resembled one or more memory locations. Whenever the microprocessor wrote into these memory locations, characters appeared on the screen. The convention used on all modern microcomputers when converting from bytes to characters is the American Standard Code for Information Interchange (ASCII).

The cathode ray tubes are scanned in a raster like a television picture. Each scan line is modulated into a series of dots called picture elements (abbreviated to *pixels* or *pels*) and each character is built up from these pixels. It soon became possible to manipulate the pixels individually so that as well as characters, dots, lines and shapes could be displayed on the screen. Microcomputers are now available with graphics capabilities rivalling those found on mainframe systems, but at a fraction of the cost. Clearly, more memory locations have to be put aside for graphics displays. For example, compare the text (character) display of the IBM Color/Graphics display with its 'high-resolution' monochrome graphics mode. The 80-character mode puts 25 rows of 80 characters on the screen. Each character is stored in two bytes – one for the character itself and one for its 'attributes', that is, colour,

intensity, etc. The whole screeen can be stored in $80 \times 25 \times 2 = 4000$ bytes. In contrast, the graphics display puts 200 rows of 640 pixels on the screen. Each pixel corresponds to one bit in memory (if the bit is 'one', the pixel is white; if the bit is zero then the pixel is black). The whole screen therefore requires $640 \times 200/8 = 16000$ bytes. Coloured graphics require even more memory, since the colour (and possibly also intensity) information has to be stored as well. This caused problems for some of the early graphics microcomputers. For example, the Acorn BBC microcomputer used an 8-bit Mostek 6502 which has a 16-bit address bus and can therefore address 64 kilobytes of memory. It had 16 kilobytes of ROM holding its operating system program and another 16 kilobytes of ROM holding the BASIC programming language. Of the remaining memory (32 kilobytes of RAM), up to 6.25 kilobytes were reserved for the computer's own use and up to 20 kilobytes were used to store the screen image. So, in the extreme case, there would be only 5.75 kilobytes of memory left for the user's program and data. This problem has been overcome in modern machines by giving the display its own dedicated memory and processor separate from the main processor and memory.

Graphics output can also be sent to a printer or plotter. Most of the cheaper printers are 'dot matrix' printers in which the print head has a series of vertically aligned pins which press through a ribbon onto the paper. In most cases, these pins can be individually manipulated to produce pixel-like rastered output. The newer laser printers can produce graphics output of a very high quality, but special software is necessary to define the complex laser path for an entire sheet of paper.

INPUT DEVICE

The usual input device is a keyboard which may contain 90 or more keys. For the novice computer user, simply finding the correct key can be a daunting task, so alternative input devices have been developed. These include touch-sensitive screens, tracker balls, light pens and so on. Perhaps the most common alternative to the keyboard is the *mouse*. This is simply a small box with one or more buttons on the top and a tracker ball on its underside. The mouse is connected to the computer via a single cable. As the mouse is moved over a flat surface, sensors detect the movement of the ball and the computer can calculate the position of the mouse. An arrow or similar symbol is usually displayed on the screen and this symbol moves in synchrony with the mouse. The mouse can be used to move the symbol to indicate choices on the screen and these can then be selected by pressing one of the buttons on the mouse. While experienced computer-users sometimes complain that mouse-based

selection systems 'get in the way', there is no doubt that they greatly simplify the operation of computers for beginners and go some way towards removing their initial trepidation.

In some applications, such as self-marked multiple choice tests for large classes, it is important that the students should not have direct access to files containing the questions, the answers or the scores. All that is required is a simple system of marking their work and here devices such as optical mark-sense card or document readers can ensure simple, secure and fast input. These devices can detect marks made in pencil or pen on specially-printed cards or sheets. Each card or sheet read can contain the responses to dozens of questions and can be read in less than 1 s. Very large classes can be handled using a single microcomputer [3, 4].

MASS STORAGE

In order to run a user's program, it must be present in RAM. However, the contents of RAM are lost when the microcomputer is switched off, so some form of long-term storage is needed. Almost all microcomputers are supplied with magnetic disc drives. These allow the microcomputer to store programs and data on magnetic discs and recall them into RAM when required. There are basically two types of magnetic disc, *'floppy discs'* and *'hard discs'*. The floppy discs are removable and can store up to 1.5 megabytes. There are two main types in use with microcomputers. The older type has a disc of magnetic material 5.25 inches in diameter inside a protective flexible paper jacket. The more modern type has a magnetic disc 3.5 inches in diameter inside a rigid plastic jacket. (They are still referred to as 'floppy discs'!) The newer, smaller discs usually have a higher capacity than the larger, older ones. The technology for making these discs has now reached the stage where most of them are sold with a 'lifetime guarantee'.

Much larger amounts of information can be stored on hard discs. These are sealed units which are usually permanently built in to the microcomputer. They can hold between 10 and several hundred megabytes and can access the information much more quickly than can be done with floppy discs. One company has recently introduced 'hard disc packs' which are effectively removable hard discs. These combine the portability of floppy discs with the storage capacity of hard discs.

Even more information can be stored on optical discs. The information is stored as tiny pits burnt into the disc material with a laser. The pits can be detected using a lower-powered laser. However, at the time of writing, this information cannot be altered once it has been burnt onto the disc. Some

optical discs are similar to the compact discs used in audio recording. These are sometimes called CD-ROMs (compact disc read only memories), and they can hold 500 megabytes or more; CD-ROM drives are now available. Larger discs equivalent to video discs can hold gigabytes of information. They can hold video images, computer data or a mixture of both, and offer great potential for the development of teaching material.

Another type of optical disc is the WORM (write once, read many) disc. Normally, optical discs need to be specially 'mastered', *i.e.*, the information has to be burnt onto them by a specialized process. With WORM discs, this mastering can be done quite simply by the WORM drive itself. However, once again, information cannot be changed after writing it to the disc.

New types of optical disc will soon be available where the information can be altered. We will then effectively have gigabytes of mass storage available to microcomputers. The question then will be what to store on them and how to manage such vast amounts of information.

CHOOSING A MICROCOMPUTER FOR TEACHING

Before choosing a microcomputer for teaching, it is important to decide exactly what use is anticipated for it. If it will be used as a stand-alone machine running published tutorial software, then the graphics capabilities of the machine and the amount of software published for it will be important. If more than six machines are going to be used, then linking them via a local area

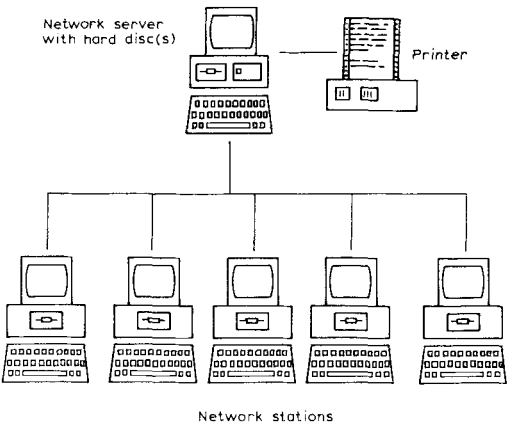


Figure 4.2. Components of a microcomputer network.

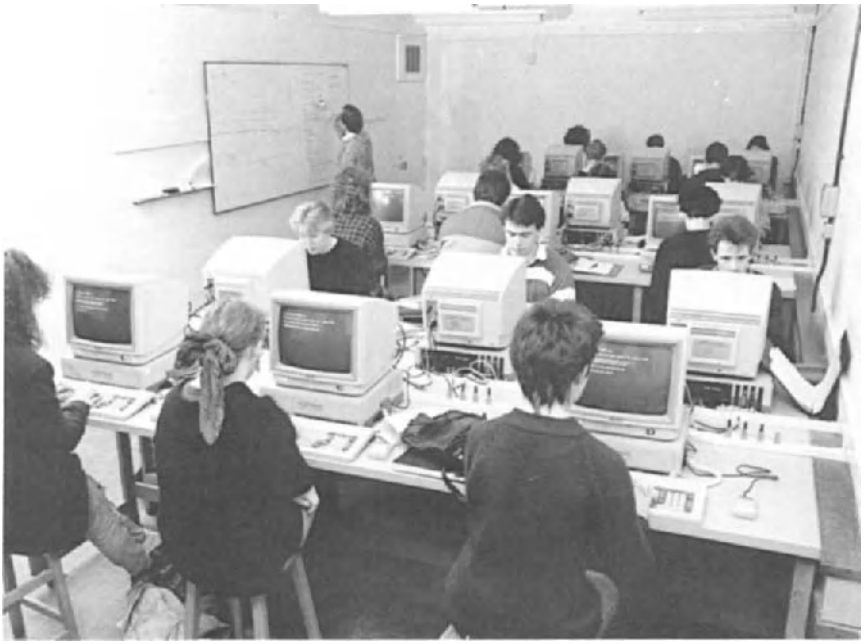


Figure 4.3. A teaching network in use. The students are working at individual stations using teaching programs downloaded from the file server which is housed in an adjacent room.

network is a good idea. In this case (illustrated in *Figures 4.2* and *4.3*) the microcomputers are all linked to one central microcomputer called the *file server*. The file server is equipped with hard discs and its function is to pass programs and data from the hard discs to the individual microcomputers (called *'stations'*) on request. Only the file server need have large amounts of mass storage, dramatically reducing the cost of storage per microcomputer. In addition, the file server or another microcomputer may provide printer facilities for the stations. The reliability of networks varies considerably and usually has no correlation with the microcomputers' other qualities such as graphics. We have had experience of two local area networks of British-built microcomputers, both of which were sold on the basis of their good graphics. While we are very pleased with our current network system, the previous one could only be described as completely unreliable. If a local area network is not going to be used, then the machines will presumably use floppy discs. If students are to have access to these, it is worth remembering that 3.5 inch discs are much more rugged than 5.25 inch ones.

Another factor to consider is whether the microcomputer will ever need to be upgraded. There seem to be two different philosophies among the manufacturers of microcomputers. The first acknowledges that the user might want to configure the computer to meet his own particular requirements and the manufacturer provides expansion slots for him to put in whatever hardware add-ons that he wants. The second group of manufacturers believe that their product provides all of the facilities that the user will ever want and therefore provide no expansion facilities at all. A good example of the second group would be the early Apple Macintosh computers. These had very good graphics (albeit monochrome) and a superb mouse-based user interface. On the other hand, the IBM-PC had a generally lower specification, but several expansion slots allowed improved graphics boards, accelerator cards, and so on to be fitted. It is now generally acknowledged that the 'closed architecture' of the Apple Macintosh was a distinct disadvantage and more recent Apple computers have expansion facilities.

A last point is that, if in addition to using the microcomputers for teaching, students use them for other purposes such as word processing, statistics or spreadsheets (see *Table 4.1*), they will be receiving a training in the use of computers which will hopefully assist them in their later careers. If an obscure non-standard microcomputer is selected, no matter how good its graphics or how fast it runs, then the students will have been done no favour. There is more software written for the IBM-PC and its clones than for all of the other microcomputers put together, simply because of the number of these machines that exist.

GENERAL PRINCIPLES

The microcomputer has the potential to increase the efficiency of both the teaching process and the learning process (*Table 4.1*).

At a time of diminishing resources and increasing staff/student ratios, teachers will obviously be looking for ways of improving teaching efficiency, if only to maintain the *status quo*. Conversely, the optimization of the learning process makes the most effective use of student time and effort.

In the case of the former, there are many ways in which the teaching process might be made more efficient, for example by using computers for administration (marking and assessment, maintenance of records, preparation of handouts and audiovisual materials), thus releasing staff to teach by more traditional methods. In this respect, there is a great deal of commercial and/or business software available, and it remains only to select what is most appropriate.

In many instances, there is nothing to be gained from writing one's own programs. Existing software will often suffice, but the problem is often one of simply finding out what is available. Databases of teaching software are now becoming available, most notably in the U.K. under the aegis of the Computers in Teaching Initiative Support Service, and these will hopefully prevent a lot of effort going into the 'reinvention of the wheel'.

In other cases, however, the novelty or speciality of the teaching task may make it desirable or even essential to start from scratch. Suffice it to say that, as a general principle, such programming should be done by individuals who know exactly what they want to achieve and who are sufficiently professional in terms of programming skills.

The same sort of principle applies to designing computer-assisted learning packages with the added proviso that the individual(s) writing the programs must have a clear conception of their educational objectives. Rather few commercial packages are available in biochemistry and physiology at present (although the number is increasing) and few individuals have the appropriate combination of skills, that is, both programming ability and knowledge of educational methods combined with a specialist knowledge of the subject area being taught. An intermediate position is provided by authoring packages which allow the experienced teacher to develop teaching material without the necessity for high levels of programming skills.

From the students' point of view, a knowledge of computers and programming should not be required. (It may, of course, be desirable for all science students to have such knowledge as a matter of principle these days, but this is a different educational objective from the one being discussed here.) In the learning situation, the only thing that should be asked of the student is that he (or she) should have sufficient manual dexterity to enter answers (for example, as choices from a menu or as numerical values) from a standard keyboard. In any case, the use of a pointing device such as a mouse can eliminate much of the need even for simple keyboard operations. It cannot be emphasized too strongly that the student should not be intimidated by the computer either in terms of complicated keyboard operations or by the stupidity of the computer (or rather its programmer). For example, in programs submitted for refereeing, we have often noticed error messages such as 'error 234 in line 4567' and which are useless to the student. Worse still are programs that crash when an inappropriate value for some parameter is entered.

At all times, it is important that the educational objective be kept in sight. In many cases, the author is carried away by the elegance of his programming and produces beautiful programs whose teaching effectiveness is less than

material produced with the simplest authoring system or even less than textbooks. This might be described as computer-aided teaching without computer-aided learning! The situation has not been helped by the attitude of some publishers, who have taken a 'shotgun' approach to teaching software. Rather than select and referee programs, they have published all of the software that they have received in the hope that the occasional program might prove to be a gem. These publishers have put poorly refereed and tested programs onto the market. Fortunately, they are easily recognized by the quality of most of their products. When buying published biomedical teaching software, it is important to remember that there is a lot of chaff and not much wheat at the present time.

Nonetheless, if properly refereed, commercially published programs are available and suitable for incorporation into a course, then this is certainly the easiest and, in the long term, the least expensive method of implementing computer-aided learning. However, sooner or later most course organizers will find that they have exhausted the ways in which existing programs can be incorporated into their courses. As courses evolve, they will include new material for which no computer-based teaching materials exist. At this point, there is a need for the production of material specifically tailored to the needs of individual courses. This need not necessarily involve high-level programming. Authoring systems offer a means of producing computer-based teaching material without having to get to grips with learning a programming language [5, 6]. They are designed to allow persons with little or no programming ability to create teaching material as quickly as possible.

At their simplest, authoring systems can provide a preprogrammed 'shell' for multiple-choice tests where the questions and answers can be entered and/or edited by the course organizer. More sophisticated authoring systems allow a variety of formats for the questions and some degree of flexibility in the way that students can enter their responses. Some systems can make use of the graphics capabilities of the computer. Perhaps the most interesting (and usually the most expensive) authoring systems are those which can make use of visual images stored on optical discs. Examples of modern general-purpose authoring systems are PILOT, TOPCLASS and MICROTEXT. There are even a few that are specifically designed for biomedical teaching, such as QUTOR.

Even with the best authoring systems, flexibility is limited. Although publishers will have tried to maximize their applications, it is not possible to conceive every possible application in every discipline. Furthermore, there are certain applications, such as the simulation of practical procedures, where authoring systems simply cannot cope with the complicated algorithms in-

volved. For these applications, there is probably no substitute for handcrafting them using a high-level programming language.

The choice of programming language will be dictated by several factors. The availability of suitable compilers or interpreters for the computer that is intended to be used will to a certain extent dictate the choice. There is no point planning to program in 'C' if a 'C' compiler is not available. The cost of the compiler or interpreter may have some influence, too. BASIC interpreters are often supplied with microcomputers and it was the microcomputer boom that caused the upsurge in the use of this language. Most BASIC programs are written as text which is stored in a tokenized or specially-compressed form. When the program is run, a special program called the interpreter translates the program word by word or phrase by phrase. Some versions of BASIC are better than others in supporting graphics, file handling, supporting structured programming, and so on. BASIC is easy to learn, but the time overhead introduced by the interpreter tends to limit its usefulness in applications where speed is essential, such as in animation. However, the interpreter does allow programs to be developed and tested very easily.

On those computers that use the MS-DOS operating system, Turbo Pascal has become very popular. This dialect of the language Pascal provides a beautifully structured environment for program production. The programs are written using the provided editor (similar to the word processing program WordStar). The compiler then converts the text of the program into the computer's built-in language (machine code) which can then be executed very quickly. Turbo Pascal offers good graphics support and file handling. Its speed of compilation (over 1000 lines of source program per minute) allows programs to be written and tested relatively quickly when compared with other compiled languages.

More and more microcomputers are now being supplied with 'graphics environments'. These all have their origins in work done at the Xerox Palo Alto Research Centre. The graphics environments provide a visually attractive interface between the user and the computer. Wherever possible, the keyboard is not used and a pointing device such as a mouse is used instead. At strategic points on the screen, there are small drawings of objects which act as 'metaphors'. For example, there might be pictures of document folders and a picture of a dustbin. The folders are metaphors for disc files and the dustbin represents a means of erasing or disposing of the files. These little pictures are called *icons*. The position of the mouse is usually shown on the screen by an arrow which moves around the screen as the mouse moves. By moving the arrow over one of the folder icons and then pressing one of the buttons on the mouse, the icon can be 'picked up'. When the button is kept

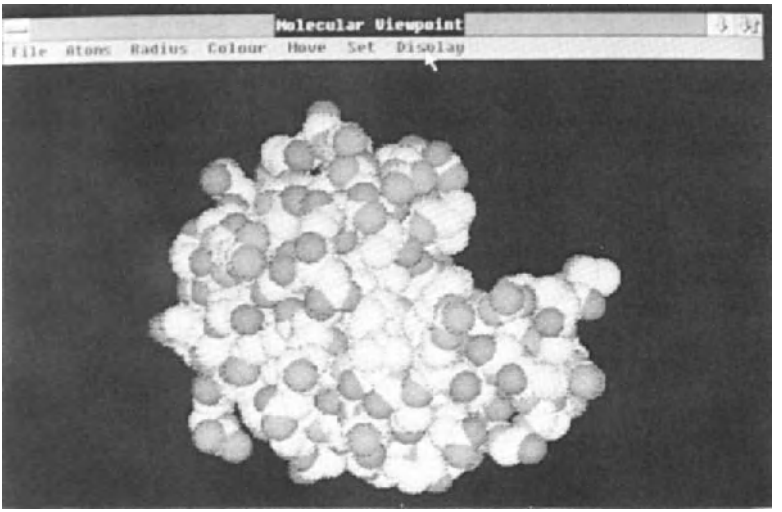


Figure 4.4. A computer-generated model of the enzyme lysozyme, produced by a program running under MS-windows.

pressed while the mouse is moved, the folder icon will follow the arrow across the screen. When the button is released as the folder icon is over the dustbin icon, the file is erased. At first, it might be thought that it would be much simpler to type in a command such as 'erase myfile.doc', but this assumes a knowledge of operating system commands, while the visual metaphors do not. The graphics environments are ideal for use by computer-naïve students. Typical graphics environments that are supported on several machines include GEM (Graphics Environment Manager) from Digital Research and MS-Windows from Microsoft. *Figures 4.4* and *4.5* show programs running under MS-Windows. Some machines, such as the Apple Macintosh, have their own graphics environments.

These environments offer the potential for very attractive interactive teaching applications, but it is not easy to write programs for them. In particular, to make the best of MS-Windows requires programming in 'C', and with its message-based multitasking it is not a system for the novice programmer to take on lightly.

Ultimately, the choice of language will be the one with which the software developer feels most comfortable and which allows him or her to produce programs most easily. As Mark Whitehorn has pointed out [5], if you are

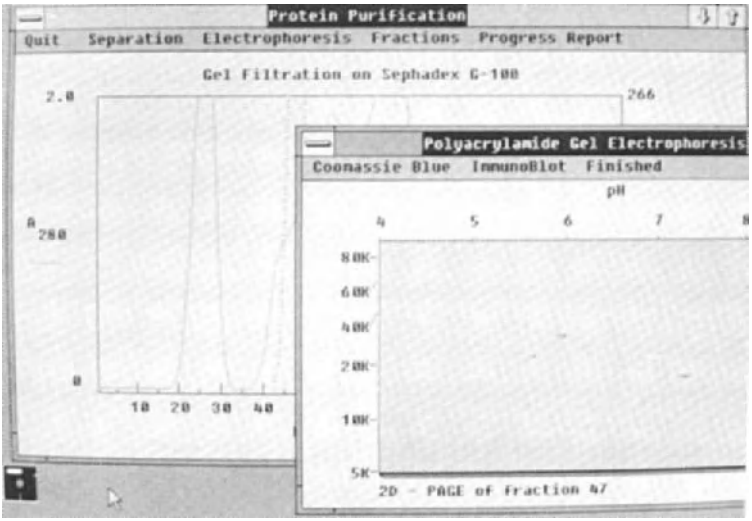


Figure 4.5. A program simulating the techniques of protein purification. This program runs under MS-windows.

actually producing usable teaching programs and you are satisfied with your programming language, then that language has passed the only two tests that are important.

THE MICROCOMPUTER TUTORIAL

There is little doubt that learning in a one-to-one situation is effective: the Oxford and Cambridge colleges have done it for many years. Among the reasons why it is effective are that the whole of the tutor's attention is focused on the individual and that of the individual student on the tutor, the environment is comfortable (that is, the student does not feel threatened or exposed when asked questions), and there is instant feedback (correction, correlation, assessment). These sorts of attributes are to some extent achievable in a computer tutorial and there are the additional benefits that the student may go at his or her own pace whilst the computer never tires and is in principle available night or day. In a way, a stage in the development of this process is the audiotutorial pioneered by the group in the Medical School at Dundee University [7]. Whether all the possible attributes of a computer tutorial have so far been achieved or properly exploited is another matter. However, the

acceptance of these sorts of objectives has consequences in terms of choice of computer and choice of software as well as for those who are actually writing software. In discussing this aspect of the educational use of computers, we will try to focus on the special advantages of microcomputers.

Irrespective of the choice of computer being used, it is essential, as already discussed, that the educational process does not require of the student any knowledge of computers or computing. Any demand for such knowledge will be intimidating and counterproductive. Because of the way in which computers work, asking a student to type in his answer to a question is fraught with problems. When asked to choose an amino acid, it is most frustrating if only the 'correct' name is acceptable to the computer. For example, if the answer were 'serine', it is a pointless exercise if the computer will not accept 'SERINE', or 'ser', or a misspelling, or 'I think the answer is serine', simply because the program only accepts 'serine' (in lower-case letters) by comparing this set of characters with what it has been instructed in the 'correct' response. Humans are, of course, much more flexible in the responses they accept. Perhaps the 'fifth generation' of computers will be equally flexible. For the present, what has been described above is a problem, especially with computers with smallish memories and without, for example, the programming facility to pick out the word 'serine' from a short sentence. The way round this is to present a list or *menu* of possible answers, designated by A,B,C,... and so on, so that the student only has to press the correct single key or move an arrow using the mouse. This probably represents the state of the art with microcomputers at the present time, although it may be justifiably argued that having to think of the answer (in a synthetic way) is not the same as picking a correct answer from a list. The same objection of course applies to any sort of multiple-choice testing. In the extreme, students may be totally lacking in both grammatical and synthetic attributes, both of which they will in fact require if they are to develop properly.

Similar problems arise when, for example, in the course of a calculation or simulation, the student is asked to enter a number. In this situation the program must contain 'error-trapping' routines, that is, the program must not hang up if a silly number is entered such as a negative pH or a very large or very small quantity. Such entries may result from inexperience with the keyboard or simply from scientific inexperience or lack of knowledge. In a human-to-human tutorial, the student would be told that, for example, "3×10^{17} is an unreasonable value for the molecular weight of a protein". The interpretation of the word 'unreasonable' in this sentence gives rise to discussion which will deal with the student's lack of experience and increase his knowledge and grasp of the area under discussion (that is, the student should

learn from the error). A computer's response could be very peremptory – "Only values in the range $10^4 - 10^7$ acceptable" – without saying why. Presumably the good student would make a note and pursue this: the poor student may just be discouraged. At the other extreme, a more imaginative (that is, imaginative in the sense of having been designed to be prepared for all possible responses – not an easy task) program might lead to a digression with positive educational effects. The student might be asked to say how many (that is, what range) amino-acid residues are found in proteins, be told if he does not know, and then be asked to estimate the average molecular weight of an amino-acid residue, again being told if he does not know, and eventually he might calculate a range of molecular weights for proteins. This is the sort of way in which a human tutor would interact with the student, but it requires a great deal of insight and forethought, and not a little work, to build this into a program. At the present time, therefore, the microcomputer is a rather poor, if tireless, substitute for a diligent and imaginative human tutor. No doubt, future advances in intelligent computer construction and programming will change this. For the present, it is better to bear these points in mind whilst considering the more positive aspects of the computer as tutor.

The second aspect of writing programs for teaching purposes involves the imaginative use of the microcomputer's facilities rather than the use of them simply because they exist. Many early so-called teaching programs simply put on the screen what could have been printed in a book and the student was expected to read (and learn) and then turn the pages electronically. The use of a computer in this way, as a book, is actually worse than a book. It is obviously more expensive, is more difficult to read (often made the more so by garish highlighting and flashing of key words), and cannot reproduce the diagrams and photographs typical of a modern textbook in the biochemical area. There is no reason to believe that such programs were any more effective than books, and they were probably less so. In order to achieve effective learning, the student must participate in the learning process. Good teachers encourage this all the time by stopping and asking questions, using models and other audiovisual aids, requiring the students to do little calculations, and so on. If microcomputer programs are to be effective in teaching, they must emulate the effective teachers and use *all* their facilities. Early programs went a small way along this road by providing frequent multiple-choice test questions, the student not being allowed to proceed to the next section until satisfactory answers were given.

SIMULATIONS AND MODELLING

Computers can be used to model systems given some information about the system. Anything from traffic flow in a town to the flux through a metabolic pathway can be modelled [8, 9]. Computer modelling of molecular structures is becoming increasingly important in biochemistry, molecular biology and pharmacology (*Figure 4.4*). Here the computer is used to carry out the matrix transformations needed to generate the required views of molecules [10–14]. It is interesting to note that in the field of biophysics, where computers are used perhaps more frequently for modelling (for example, for fitting X-ray diffraction data to predicted structures) than in other subjects, to our knowledge very few teaching programs have so far been produced.

In other applications, it is possible, given a certain amount of information to start from, to try to find unknowns by testing values for an unknown parameter until a model behaviour is achieved that matches what is observed in the real phenomenon. Indeed, there are programming languages such as PROLOG which are specifically designed for this sort of task. Any computer can be used for this sort of investigation, but microcomputers are becoming increasingly useful in this field as their speed and memory capacity increase. This is the research aspect of using computers and, while it is obviously good for students to learn of the potentialities for this purpose, any such research is likely to involve high-level programming skills. In this article, we are concerned with the educational (and administrative) uses of microcomputers and we wish to highlight the potentialities of computer simulations for educational purposes and emphasize once again that this should not require any programming skill on the part of the student.

In addition to the increasing speeds and capacities of modern microcomputers, their outstanding graphics capabilities give a further impetus to using microcomputer simulations for teaching purposes. It is first necessary to consider the difference between a computer tutorial and a simulation. We will then go on to consider the advantages of simulations and give some examples of their successful use.

In fact, computer tutorials and simulations overlap, but in general a tutorial carries the implication that the student is led through a series of steps–experimental evidence, and logical steps – with testing and correction, if necessary, until the lesson is learnt. In contrast, a simulation program allows the student to go where he will and to learn by experience, including making mistakes. This requires that all parts of the simulation programs are accessible in any order. There may be the additional facility for storing data at a given point so that the student can continue at another time, possibly having done a laboratory experiment or gone to the library in the meantime.

It is important to be clear that a simulation of an experiment does not replace laboratory experiments. Subjects such as biochemistry are experimental sciences and, in general, discoveries are made in the laboratory with the requirement for manual laboratory skills. However, part of the skill of successful laboratory work involves not manual skills, but skill and expertise at designing appropriate experimental strategies. In addition to the various reasons for considering simulation packages as educational tools mentioned below, a simulation can be used to teach strategy, for example, how to go about carrying out restriction mapping of a piece of DNA. In this case, the computer would hold the pertinent information on restriction sites so that, given a DNA sequence, the sizes of the fragments produced by a given restriction enzyme can be calculated and displayed, either as a sequence or, more appropriately, as positions of bands after agarose gel electrophoresis. We now see the immediate advantages of the microcomputer. The machines are cheap enough for them to be available to students, and their graphics capabilities allow the presentation of the calculated data in the form of electrophoresis data, chromatography column elution patterns, or whatever.

In addition to teaching strategies by trial and error, what then are the advantages of simulations? These may be summed up by saying that simulations allow students to carry out 'experiments' that are too time-consuming, too expensive or too dangerous to be actually carried out in a class practical. Although strategies will be learned and students will gain experience, for example, of what it is sensible to do to make progress in solving a problem, laboratory experience will be required, too. However, the extent to which this is necessary for the professional development of the student as a scientist may vary. Thus, medical students, few of whom are likely to become laboratory workers in their professional careers, may need to do relatively little laboratory work but be more concerned with trying values for different parameters, interpreting data, dealing with statistical variation, and so on. In contrast, for students destined to become professional biochemists, it may be that the learning of skills in devising strategies for problem solving can be accelerated by simulation techniques. For example, in order to determine the various parameters for a given enzyme (K_m, V_{max}, and so on), it will be necessary to carry out a large number of rate determinations of the enzyme activity under different experimental conditions, with suitable duplicates and controls, but all basically involving the same manual experimental skill such as pipetting reagents into a cuvette and taking readings from a spectrophotometer. While it may be appropriate for a research student to actually do this in the laboratory, it is much more difficult to justify it for a B.Sc. or first degree student where enzymology only forms a small part of the course and laboratory time

is limited. Having understood the basic experimental determination in the laboratory, the student can move to the computer to study the effect of varying substrate concentration, can look at the various ways of transforming and plotting the data and can be made aware of the effects of statistical variation on the accuracy of the enzyme parameters it is desired to determine. Such a program is provided in ENZPACK [15] and a similar type of program is reported by Dahmer [16].

In this example, the appropriate series of laboratory experiments would take a long time, which may make it not feasible to put on in a practical class. In other instances, such as genetics experiments, the experiment itself might take an extremely long time, or in the case of human genetics, might not actually be possible. The simulation can then illustrate what might happen, demand an interpretation of data obtained (again with appropriate statistical 'noise' added by the computer), and suggest to the student what he might look for in the available real data or how to plan an experiment or a survey.

The protein purification simulation program that has been developed in Leeds ([17], shown in *Figure* 4.5), illustrates some of the aspects of the use of such programs. The scenario is that the operator has to purify one enzyme protein to homogeneity from a mixture of 20 proteins by choosing methods, in an appropriate sequence, from those offered – ammonium sulphate fractionation, heat treatment, ion-exchange chromatography, gel filtration, chromatofocusing and so on. The program development started with a search of the literature to obtain as complete as possible data for a number of enzymes, including the M_r, subunit structure, amino-acid composition, heat stability and hydrophobic–hydrophilic balance. These were stored and then algorithms devised to calculate the behaviour under given experimental conditions. Thus, when the operator chooses to carry out ion-exchange chromatography on DEAE-cellulose, it is necessary to work out how each of the proteins would behave on a column at a given pH and ionic strength, and then add these together to produce an appropriate elution pattern. The graphics capability of the microcomputer was utilized to display such an elution profile, to locate the enzymic activity and thence to offer the possibility of pooling fractions and examining the pool by one- or two-dimensional electrophoresis, again displayed on the screen (*Figure 4.5*). The separation steps can be chosen in any order. At each step a purification table can be displayed of specific activities, yields, and so on. An account is given of the expense: preparative isoelectric focusing is much more expensive than ammonium sulphate fractionation both in time as well as in materials used.

Such a program is not intended to replace the gaining of real experience of working in the laboratory: it is intended to be used in addition to such

experience, to make the student understand the principles behind the separation methods, which he might have to go away and look up, and to develop practical strategies for how to proceed. In some ways it is a game, but it is nonetheless a game of skill. Experienced hands at purifying proteins can get a protein to homogeneity more rapidly and more economically than novices.

Ultimately, the accuracy of a simulation will depend on the accuracy of the information built into it. Each simulation therefore has to be a miniature 'expert system'. Good examples of this are the diagnosis programs of Blanchaer [18], in which the students have to diagnose and then treat 'patients' supplied by the computer. These were originally devised as 'hidden text' booklets: consequently, the system and flow schemes for many of the examples had already been worked out before being converted for computers. A great advantage of this type of program is that, unlike the real world, the students can make mistakes (and hopefully learn from them) without the risk of harming patients or getting involved in litigation.

MANAGEMENT OF EXAMINATIONS

The development of a computer or a program that can mark an essay-type question, even at GCSE level, is still some years away. In contrast, multiple-choice type questions lend themselves very readily to computer marking either directly from the keyboard, or, more usually, via mark-sense cards [19]. In between these two extremes, there are various possibilities, such as producing responses (that is, 'correct' answers, textbook references) to multiple-choice tests, or of moving toward computer-marked modified essay questions. In addition, when dealing with large numbers of students, the computer can help in the administration in various ways from selecting questions at random from a bank to printing lists of marks or grades. There is nothing especially new about this in the field of biomedical education. Most of the things described have been done on mainframe computers for years. The coming of the microcomputer simply means easier access of students to computers and such things as portability.

In what follows, we describe briefly what can be done with multiple-choice testing, how it can be extended, and how the microcomputer can be used in test administration (*Table 4.2*).

Table 4.2. USE OF MICROCOMPUTERS IN EXAMINATION ADMINISTRATION

(1) Select questions from question bank either completely at random, or on parts of the syllabus, or to cover all parts of the syllabus.
(2) Print or word-process the test papers or master copies for printing or duplicating.
(3) Receive input from marked-sense card reader and process the incoming data, that is, mark the test.
(4) Produce a list of names and marks in whatever order desired and produce a histogram.
(5) Perform statistical analysis on the results, for example, difficulty indices.

MULTIPLE-CHOICE TESTS

Few students actually like doing multiple-choice tests, but both they and we, the teachers or assessors, have come to accept that for the administration of large classes, they are here to stay. Certainly, when properly written and used, they can form an objective and reliable measure of certain components of cognition, especially recall of factual information. Writing multiple-choice questions that are unambiguous or free from false detractors, is very difficult and demanding, but has nothing to do with computing. Having decided on what the answers are, the computer can very rapidly mark and grade via a mark-sense card reader.

In the U.S.A., the Association of Medical School Departments of Biochemistry runs a Question Bank [20] which in 1981 had approximately 5,000 questions in it and which was used widely in U.S. medical schools. The history of this question bank has been described [20]. The questions themselves ranged from single-answer multiple-choice, to matching, including fill-in-the-blank, as well as essay, but of course only the first two types can be computer-graded. What is interesting about the account given by the authors [20] is that they had already perceived the future potential importance of microcomputers. Although their basic output was on 'industry standard' magnetic tape, they could also distribute copies on the now obsolete 8-inch discs (for example, for a PDP 11 or for microcomputers). As early as 1981, they had begun to develop programs in Pascal and were also using an Apple III. They mention that they would ultimately "develop programs that will enable a student to use a microcomputer for study-self examination and testing".

Various texts now aim to give American medical students direct experience of answering questions of the type that are in the Question Bank, including

the second edition of Devlin, Textbook of Biochemistry with Clinical Correlations [21] and Lippincott's Illustrated Review of Biochemistry, [22].

For computer-marking, the student simply chooses one (or more) correct answers from, say, five questions or possibilities, and the computer compares these choices with what it has been told are the correct responses. Many variations on the theme are possible, for example, "of four statements and possible responses, check *a* if all four are true, *b* if 1,2,3 are True and 4 False,... until *e* all are false," but always the response will be a small set of figures or numbers. (It may sometimes seem appropriate to give students extra marks if they can understand how to answer the questions!)

Any computer can check such answers, and the only advantage of a microcomputer is that usually cards can be put through at any time with a dedicated set-up (rather than the cards having to be put through in a batch and the data processed all at the same time). This latter arrangement is reasonable for examination, but poor for individualized self-assessment over the academic year.

In Leeds, we have for some years been using an inexpensive system which is based around a Chatsworth Data Corporation optical mark-sense card reader. An early form of this system used an Acorn BBC microcomputer [3], but this was later replaced with an Apricot F1, a 16-bit machine running MS-DOS. The entire system is contained in a cabinet which also houses a colour monitor and a dot-matrix printer. The cabinet is mounted on heavy-duty castors and can therefore be transported to wherever the tests are to take place. This is usually a teaching laboratory, where our students mark their own self-assessment tests (*Figure 4.6a,b*). They receive instructions from the monitor and receive an individualized output from the printer (*Figure 4.6c*). This output gives them their score and for each question that they have not scored completely correctly it gives a paragraph of information indicating the nature of the error, what the correct answer is and, where possible, page references to three textbooks.

The cards are 3.25 inches wide and 11 inches long. Each has a header region where the student marks his identity and the identity of the test being marked. The remainder of the card is used for indicating responses. Each card can hold the responses of up to 42 questions, arranged in six banks of seven lines (*Figure 4.6d*). Each line can have up to nine choices, giving us great flexibility in the format of our questions. In addition, a tenth position can be marked to indicate 'don't know'. If this position is marked, all other marks for that line are ignored and the remedial text is automatically given.

This system now works perfectly and has been well accepted by the students. However, during its development, we had to incorporate error-trappig

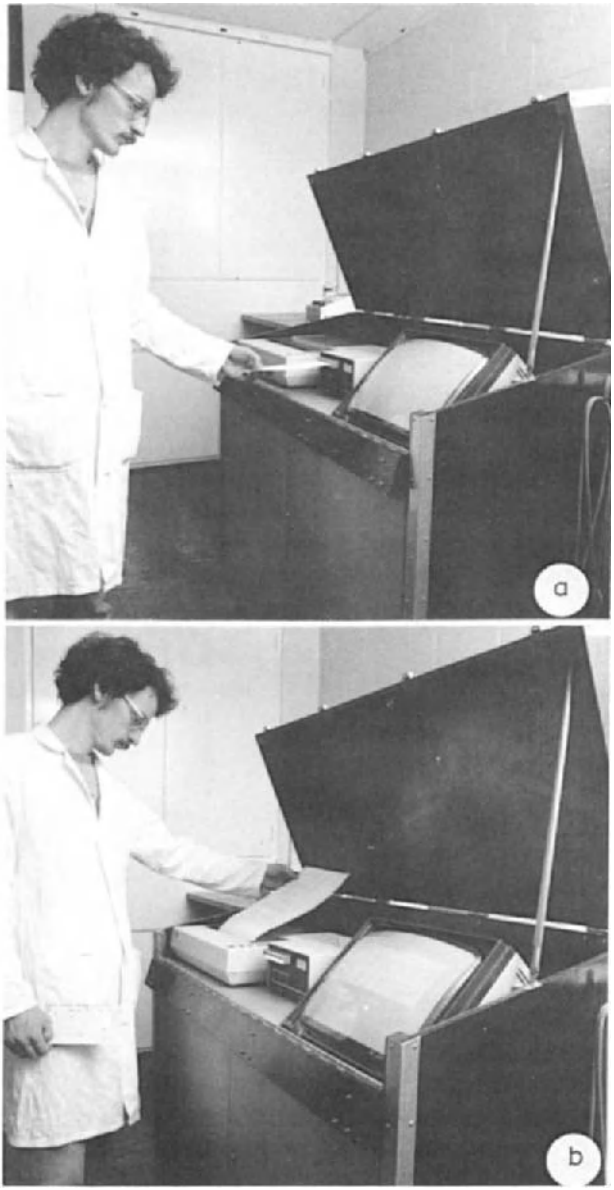


Figure 4.6.(a) The computer-marked MCQ system. The hardware is housed in a portable cabinet. The computer, the discs and the paper are housed inside the cabinet and the doors are kept shut during the test. The students have access to the monitor, optical mark-sense card reader and printer in the upper compartment. (b) The MCQ system in use for student self-assessment. (c) Part of a typical output sheet. (d) Part of a card on which the students record their responses.

(c)

UNIVERSITY OF LEEDS
DEPARTMENT OF BIOCHEMISTRY – TEST AC
Page references are to the second editions of Stryer and Lehninger and to "Principles of Biochemistry" by Lehninger

A.NOTHER Student No. 330
You got 8 right, 5 wrong and missed 36.
That corresponds to a score of 16.4%.

Question 1 Line 1
True.
Carbohydrates and fats are broken down to acetyl CoA which enters the Krebs cycle and the carbon atoms are released as CO_2. Thus a reduced form of carbon becomes more oxidized by dehydrogenation by NAD^+ etc. The NADH produced yields ATP via the cytochrome system.

Question 1 Line 2
all correct

Question 1 Line 3
all correct

Question 1 Line 4
False.
Plants are capable of respiration via their mitochondria just like non-photosynthetic higher organisms.

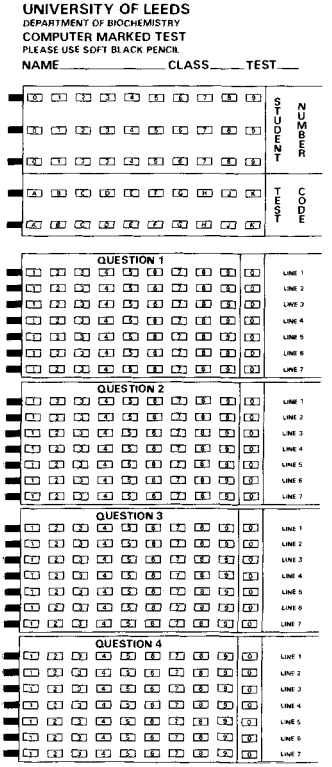
Question 1 Line 5
True.
Plants use sunlight to obtain energy for the synthesis of complex materials. They can break these down for energy at other times but ultimately they are dependent on solar energy.

Question 1 Line 6
all correct

Question 1 Line 7
all correct

Question 2 Line 1
True.
Assuming that no Krebs cycle intermediates are lost or gained, their levels will remain constant, the CO_2 given off being derived from acetyl CoA (from pyruvate).

(d)



Thus the oxaloacetate molecules are involved in an infinite number of turns of the cycle and thus act catalytically. The addition of a very small amount of oxaloacetate will catalytically stimulate the utilization of large amounts of pyruvate.
Stryer II p. 302, Lehninger II p. 448, Lehninger Principles p. 442.

Question 2 Line 2
False.
Acetyl CoA is consumed by the cycle and is released in the form of CO_2. It is therefore a substrate for the cycle and each molecule can participate in only one turn of the cycle.

into both the software and hardware. The software error-trapping is designed to reject badly-marked cards. The hardware error-trapping consists of making sure that the hardware is 'student-proof'. (A student using an early version of the system received the instruction "Put your card through the reader". Despite the fact that the reader was directly in front of him, he rammed his card into the 3.5 inch disc drive!)

The system keeps a record of each student's score for each test. However, the students indicated that they did not want lists of names and marks to be published. Instead, we provide histograms and the distribution of scores for each test. The individual student can then check his score against that of the class as a whole.

There are moves in the direction of a more literate interaction with the computer. The diagnosis programs of Blanchaer [18] have already been mentioned, and obviously examinations could take this type of format. Some years ago, the Modified Essay Question was suggested as a way of examining General Practitioners [23] and this offers a possible model for progress in this area. Indeed, the author suggested that, "A secretary, preferably medical, can often be trained to do the majority of the marking", given a response guide and marking schedule. An example of a question, in a sequence, is as follows.

> As a result of your investigations, pregnancy is confirmed. Mrs A. has clearly serological evidence of childhood infection with *Rubella* when she attends your antenatal clinic 10 days later. List the information you require in order to make appropriate arrangements for her delivery.

Obviously choices from a given list would be easy to mark, but there would be problems with information being typed in as regards format and spelling, and also, of course, the list is not finite. Other questions which finish "How would you handle this situation?" or "What action would you take at this stage?" are even more open-ended (at least as far as a stupid micro is concerned), although for an experienced GP there might be absolutely no doubt as to what to do. As ever, the problem is in the language and in preplanning for all possible responses. For the moment, all we can say is that picking out an answer from a colloquial English phase is not what microcomputers are good at in their present stage of development.

COMPUTER-CONTROLLED VIDEODISC

In microcomputer simulations, it is advantageous and a relatively simple matter to produce images–graphs and charts, 'pictures' of chromatography columns, or of protein models, and so forth (see *Figure 4.4*). These can be

manipulated to some extent; for example, graphs can be altered, models can move or change colour, so that the program can be interactive. It has been exercising the minds of a number of individuals how such programs could be combined with real images, either still or moving. Bryce [1] has described a random access microfiche system interfaced with either a mainframe or a microcomputer (Cromemco System Three), but of course this cannot produce moving pictures.

Videotape is a very inexpensive medium nowadays, in that the equipment required to play videotapes is domestic and therefore cheap. However, tape is very poor as a medium for interaction, since interaction demands rapid access to any segment of a program: searching through a tape will never do this in seconds, even if inexpensive equipment is available. The answer to this problem of instant access, at least at present, seems to be the videodisc controlled by a microcomputer. A number of domestic videodisc players are available, although this technology has not caught on to a very significant extent for home entertainment compared with videotape. Videodiscs, both domestically and in educational systems, have many disadvantages, such as high cost in both players and disc production, and the fact that discs are 'read-only' (a read-write disc at a conceivable cost for education purposes is some time away). However, they *do* offer instant (max 3 s) access to any frame on one side of a disc [24]. In one system presently in use (originating from the BBC) and which is being targetted at secondary schools, each side of the 12 inch videodisc can hold either 55,000 stills or about 35 min of moving video, with sound, or any combination of these. In addition, each side of a disc can also store 350 megabytes of data, text or programs which can be integrated with the visual information.

It should be mentioned that the still frames are of high quality, unlike those obtained with VHS videotape players on 'pause'. One use that suggests itself is to put a whole histology slide collection on a disc. Certainly there are such discs in existence (UK PATH I [25]). A disc entitled 'Cell Biology', which contains a number of short film sequences, has been produced by the Institut für den Wissenschaftlichen Film in Göttingen [26]. An interesting feature of this disc (and the facility is potentially available for all videodiscs) is the presence of two sound-tracks, one in German, the other in English. Another disc in existence is on anatomy and is entitled 'The Knee' [26].

Videodiscs run on a player which is controlled by a microcomputer which gives instantaneous access to each of the 55,000 frames individually, but obviously by going through a succession of frames or of short bits of movie, under the control of the microcomputer program, it is possible to set up interactive sequences with branch points and so forth, just as with the computer alone.

The barriers preventing increased access to such systems are the relatively poor accessibility of computer-controlled videodisc players, which at the time of writing cost about 10-times as much as a VHS videotape player, and the initial cost of making discs. In the latter case, there is to be added to the not inconsiderable filming costs, the cost of mastering the disc, copies of which would then sell at up to £200. In most cases, only large organizations will be able to get together the start-up funding for a videodisc project, and they will focus on areas where it can be expected to sell a considerable number of discs in order to recover their set-up costs (such as in all the medical schools in the world). No doubt another factor is that many organizations will wait before purchase to see what sort of uniformity there is going to be in terms of international standard formats. There is equally no doubt that, as an educational methodology, videodisc has enormous potential.

DOES CAI WORK?

The question of whether CAI is educationally effective demands an answer whether the computer system in use is mainframe or micro, and we do not propose to do more here than to indicate the sorts of data that have been collected. There have been a number of studies aimed at discovering whether CAI is better or worse than conventional teaching, setting aside any economic advantages in saving staff time. Such studies have to focus on specific objectives, the achievement of which can be tested. Rather few of the studies concern the medical-biochemical area. It may be noted that a considerable percentage of medical schools in the U.S.A. and Canada responded to a recent questionnaire that they were already using CAI or expected to use it soon, and consequently we may expect to see studies in the biochemical area appear soon. In the U.K., the introduction of computers for teaching in universities has been funded by the Computers in Teaching Initiative. A condition of the later awards by the C.T.I. has been that the effectiveness of the computer-based teaching should be assessed. There should therefore be a considerable amount of information produced which will be published by the C.T.I. Support Service.

Apostolides [28] and Escanero and Alda [29] have summarized the key literature on CAI and its effectiveness, and these will not be discussed in detail here. In his report on the effectiveness of CAI (using a mainframe computer) in biochemistry, Apostolides [28] looked at students' learning and retention, and also at their acceptance of computer teaching methods. He used three programs, one on glycolysis, one on the Krebs cycle and one on enzyme

kinetics (ENZKIN) and concluded that in the drill and practice mode as well as in computer simulation (that is, ENZKIN), CAI "...had no detrimental effect on students' academic achievement compared with conventional methods of learning". Furthermore, the students liked using the computer and would have liked more of it.

Escanero and Alda [29] reported the results of a test in which 24 students studied the Krebs cycle, 12 of them using textbooks and 12 using CAI. Both subgroups were evaluated by a standard test (a) before the experiment, (b) immediately the students thought their level of preparation was optimal, (c) 1 week after this, and (d) 3 weeks after this. The CAI students spent more time on the subject area, got somewhat better test results (after the CAI) and retained the knowledge for a longer time.

Nevertheless, much more work is needed in this admittedly difficult area. Escanero and Alda [29] mentioned that of 100 papers on CAI usage in the Health Sciences, less than 10 evaluated the effectiveness of CAI. It is difficult to arrive at a consensus of opinion at present. Many educational experiments in the evaluation of CAI, especially in the area of physiology, appeared to show zero to slight improvement in many of the cases. Set against this may be the fact that the use of computers may save staff time: in other words, the same effect is achieved but using fewer resources. Also to be taken into account are the observations that a high proportion of students believed that the computer method enhanced their learning and consequently were prepared to put more time at a given test. Clearly, the easy availability of microcomputers, that is, many machines available around the clock or at home, should enhance this process.

THE MICROCOMPUTER AS A PIECE OF LABORATORY EQUIPMENT

Each microcomputer contains at least one timer, a cathode ray tube and is capable of producing traces on a dot-matrix printer. Microcomputers can easily emulate pieces of laboratory equipment such as storage oscilloscopes or chart recorders. The microcomputer is often a less expensive option than the item of equipment that it is emulating and so equipment manufacturers are increasingly supplying, for example, a microcomputer and a dot-matrix printer instead of a multi-channel chart recorder. This opens up new possibilities for the teaching laboratory where multiple sets of apparatus are often required. It might not be possible to provide each student studying physiology with a storage oscilloscope, but would not be difficult to provide microcomputers equipped with suitable interfaces.

To be useful in this context, the microcomputer will need to be connected to the device actually providing the data. This might be, for example, a pressure transducer, a scintillation counter, or a spectrophotometer. Most microcomputers are supplied with two 'standard' interfaces. One is a *parallel* interface which is usually used to connect to a printer. It has basically 8 connections which are used to pass information one byte at a time to the printer, each bit of the byte having its own connection. The 8 bits are all transferred at the same time, hence the term 'parallel'. In addition, there are usually a couple of connections that are used by the computer and the printer to agree about when to transfer bytes across the interface. Unfortunately, from our point of view, while this interface is theoretically capable of two-way communication, the computer manufacturers nearly always wire up the parallel connector as output only. An exception to this is Hewlett-Packard. They provided an input-output parallel connector which they called the HP-GPIB (General Purpose Interface Bus). This proved to be very popular and a large amount of laboratory equipment has been built which can convert measurements into bytes which can be passed in turn to a computer via the HP-GPIB. So popular, in fact, was the HP-GPIB that the Institute of Electrical and Electronic Engineers adopted it as a standard and it is now generally known as the IEEE 488 standard parallel interface, and add-on boards that provide IEEE 488 interfaces for most microcomputers can now be bought.

The other interface usually found as standard on microcomputers is a *serial* interface. In this case, the bits of a byte are sent one after another down the same wire. The computer and the device that it is communicating with must both agree about the speed at which the bits are to be sent and received. (The unit of this speed is bits/second, called *'baud'*, and the speed itself is called the *'baud rate'*.) Since the receiving device does not know exactly when to expect a byte, the byte is usually preceded by a 'start bit' which indicates that a byte is on its way. The serial interface is provided for use with serial printers or with modems. A modem allows two-way communication via a telephone line. Hence, serial interfaces nearly always allow input as well as output and are therefore suitable for communication with laboratory devices. More and more equipment manufacturers are providing serial communication interfaces with their equipment. It is an attractive alternative to parallel communication, since it requires fewer wires (cheaper cables) and it can be used over much longer distances than parallel communication. However, by its nature, it tends to be slower than parallel communication and is therefore not suitable for the capture of rapidly-changing data. The maximum useful baud rate for most microcomputers is about 115,000 baud, although most serial communication is much slower than this, in the range 300–9,600 baud.

For some applications (such as emulating a storage oscilloscope or capturing stopped-flow data [30]), neither serial nor parallel communication is suitable. Rather than receiving data that have been converted to bytes by the sending device, the computer needs to be able to measure directly externally-applied voltages. In this case, a special interface containing an A/D chip (analogue to digital converter) is required. These compare the applied voltage to a standard voltage and express the result as a binary number. The cost of an A/D device is determined by the speed with which it can do the conversion and the number of bits in the result. Clearly an 8-bit A/D converter will only be able to divide the range of applied voltages into 256 steps, while a 12-bit converter will provide 4,096 steps and a 16-bit converter 65,536 steps (and you will pay for them!).

This is an aspect which is as yet largely unexplored. New, more relevant practical classes can be devised in which expensive items of apparatus can be substituted by microcomputers. Without doubt, this aspect of the use of microcomputers in teaching has importance for developing countries and will hopefully go some way to overcoming the depressing effects of spiralling equipment costs and lack of maintenance facilities.

THE FUTURE

Clearly, we are currently only in the early stages of the use of CAI with microcomputers in the biomedical area. At the moment, the supply of teaching programs is limited, but as projects such as the Computers in Teaching Initiative come to fruition, this situation should improve. New hardware developments such as the introduction of high-speed parallel processors (for example, the transputer) will allow affordable real-time animation for the first time. New software developments will certainly have an impact. Perhaps the most exciting is the 'hypertext' concept in which students can explore interlinked and extensively cross-referenced information systems where they can access text, graphics and so on. A good feel for hypertext is given in the review of Apple's Hypercard product [31]. The combination of hypertext with expert systems and video technology offers exciting possibilities.

Communications will become increasingly important. At the moment, teaching computers tend to be linked to each other only in local area networks (see *Figure 4.2*). In some cases, these are connected via campus mainframe networks to national networks such as JANET (Joint Academic Network). However, in the main, these wider communications are only used for such things as electronic mail. It is likely that, in the future, these links will be used

for the exchange of teaching materials and that international satellite communication systems will allow more widespread exchanges. These will become more and more important as ventures such as the Commonwealth of Learning get under way and require distance-learning materials.

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5 Applications of Modern-High-Field NMR Spectroscopy in Medicinal Chemistry

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INTRODUCTION

In 1971, one of us published a monograph which described the value of proton magnetic resonance (PMR) spectroscopy to medicinal chemists and to their biochemical colleagues [1]. Examples were drawn from results published over a period from the late 1950's, when commercial NMR spectrometers first

became available, and were confined to the hydrogen nucleus, since studies of other magnetic nuclei were in their infancy at that time. The 10 to 15 year period since that book appeared has witnessed dramatic developments in NMR technology, principally as a result of the introduction of pulse Fourier Transform (FT) spectrometers, which overcame sensitivity problems and allowed a wider variety of NMR experimentation than the original continuous wave (CW) instruments, and the replacement of electromagnets by superconducting magnets which gave access to higher magnetic fields and greater resolution.

Pulse NMR was originally developed to observe nuclei which were insensitive to CW spectroscopy. A nucleus in this category, and of particular interest to medicinal chemists for structure elucidation, is carbon. The abundance of the NMR-active isotope of this nucleus, ^{13}C, is only about 1%, and this fact combined with the inherent insensitivity of NMR and the low gyromagnetic ratio for carbon, makes this nucleus virtually impossible to observe directly by CW spectroscopy. Pulse NMR overcomes this sensitivity problem by applying a short pulse of radiofrequency to the sample, which excites all the nuclei simultaneously. The pulse causes the vector representing the net magnetization of the sample to rotate from its original position aligned parallel to the external magnetic field. The angle of rotation depends on the length of the pulse and is used to represent the pulse when describing pulse NMR experiments, for example, 90°_x, where x is the direction of the pulse in the conventional coordinate system. At the end of the pulse the nuclei relax back to equilibrium and the resulting free induction decay (FID) signal is detected over a finite period (data acquisition time) and converted to the familiar spectrum by performing a Fourier Transformation with the aid of a computer. The development of the Fast Fourier Transform algorithm allowed the use of mini-computers, which have become integral parts of the modern spectrometers, so that the FT calculation can be performed on a time-scale realistic for routine collection of NMR spectra [2]. In addition, the computer is also used to control the operation of the spectrometer. Commercial FT spectrometers first became available in about 1970. Each scan takes only a few seconds compared with several minutes for CW spectroscopy. FIDs from individual pulses can be digitized, summed until a suitable signal-to-noise ratio has been achieved, and then transformed. Descriptions of the pulse FT NMR experiment are available in basic texts (for example, [3, 4]).

Pulse NMR is not just confined to ^{13}C spectroscopy, but is now used for the study of a wide range of nuclei, although this review concentrates on carbon and proton nuclei. All modern high-field spectrometers operate in the FT mode, and the success of pulse FT NMR is due to its versatility. Quantum

mechanical descriptions of the pulse experiment have allowed the rational design of multipulse sequences which give spectra containing information other than straightforward chemical shifts and coupling constants. Great ingenuity has been displayed in devising these sequences and their acronyms! Multipulse experiments for one-dimensional spectroscopy have been reviewed in detail elsewhere [5, 6].

Examples of multipulse sequences for routine ^{13}C spectroscopy are those used to determine carbon multiplicities. The normal carbon spectrum is obtained under conditions of proton irradiation so that each signal appears as a singlet and increased intensity is achieved by the Nuclear Overhauser Effect (NOE). However, the multiplicity of carbon signals is a valuable aid to spectral assignment. This information can be restored, while retaining the simplicity of the proton decoupled spectrum, by using the INEPT (**I**nsensitive **N**uclei **E**nhancement by **P**olarization **T**ransfer) [7] or DEPT (**D**istortionless **E**nhancement by **P**olarization **T**ransfer) pulse sequence [8]. An interval between pulses in the sequences can be adjusted to give positive, null or negative signals depending on the number of attached protons (see section on ^{13}C-NMR, p. 373). Addition and subtraction of INEPT or DEPT spectra using different pulse intervals allow subspectra for the individual types of carbon to be obtained ('spectrum editing'). In addition to revealing carbon multiplicities, INEPT and DEPT enhance the intensity of the carbon signals by transfer of magnetization from the attached protons (polarization transfer).

Pulse NMR spectroscopy has simplified the measurement of relaxation times. Spin-lattice relaxation (T_1) can be determined using the inversion recovery or saturation recovery techniques [9, 10]. Selective T_1 measurements, where a single signal is irradiated, have been used to obtain molecular geometry data as the relaxation rate is inversely proportional to the sixth power of the distance between nuclei which interact by dipole-dipole relaxation, as illustrated by combination with NOE measurements (see below) in the structure elucidation of gramicidin S [11]. Internal molecular motion can be indicated by comparison of T_1 for different parts of the molecule, as used by Pappalardo, Radics, Baldo and Grassi [12] for studies of internal motions in opiate agonists and antagonists. Spin-spin relaxation (T_2) can be measured by the Carr-Purcell-Meiboom-Gill (CPMG) sequence [13, 14]. Relaxation time measurements can also be used to determine the binding characteristics of ligand-macromolecule complexation.

One of the most useful aspects of NMR compared with other types of spectroscopy is the elucidation of spatial geometry. Modern high-field instruments give spectra in which the fine structure of the signals is more clearly resolved but may still not always show first-order coupling. The analysis of

coupling constants can yield stereochemical and conformational data, examples of which are given later in this review. NOE measurements can give an indication of 'through-space' distances between nuclei, which may indicate conformational preferences and aid stereochemical assignments. NOEs are measured by saturating the signal of interest (by gated decoupling with irradiation off during data acquisition) and comparing the intensities of other resonances in the irradiated and unperturbed spectra. This comparison is conveniently achieved by 'difference spectroscopy' in which the control spectrum (usually with off-peak irradiation so that any heating effects of decoupling on the sample are the same for both spectra) is subtracted from the irradiated spectrum. Enhanced signals appear as positive peaks in the difference spectrum, examples of which are shown later (*Figures 5.5, 5.32, 5.24* and *5.43*). Many groups are now combining such three-dimensional structural data obtained by NMR for peptides and proteins with computational molecular modelling techniques to gain an insight into their conformation in solution.

TWO-DIMENSIONAL SPECTROSCOPY

The difficulty of interpreting crowded one-dimensional spectra has been tackled by the development of experiments which display spectral data in two dimensions. Some of these experiments can be difficult to appreciate fully other than in theoretical terms. However, this should not preclude the medicinal chemist from making use of the spectra obtained for the purposes of structure elucidation.

The concept of two-dimensional NMR spectroscopy was introduced in 1971 by Jeener [15] and its potential was demonstrated by Aue, Bartholdi and Ernst [16]. Jeener suggested a sequence in which an extra time interval and pulse were inserted into the usual pulse ^{1}H-NMR experiment to give 90_x–t_1–90_x–t_2.

Magnetization transfer occurs between coupled spins during the interval t_1 so that the amplitude of the signal for each nucleus is a sine function of t_1 which depends on its own frequency and the frequencies of nuclei coupled to it. In the 2D experiment, the above sequence is repeated for a series of time intervals, t_1, and the FID transformed for each as usual with respect to t_2, the data acquisition time. Then a second Fourier transformation with respect to t_1 is performed, giving a spectrum which has chemical shift data in two dimensions, f_1 and f_2, corresponding to the two transformations. The peaks that appear in the spectrum can be described by co-ordinates in (f_1, f_2). Those on the diagonal reflect the one-dimensional spectrum and occur at the same chemical shift position (ν, ν) in both dimensions. Off-diagonal or cross-peaks

occur at the chemical shift positions where two nuclei are coupled. The spectrum should, of course, be symmetrical about the diagonal, with cross peaks occurring at (ν_1, ν_2) and (ν_2, ν_1). Complete symmetry is often achieved with the aid of a computerized symmetrization routine to overcome non-symmetrical experimental artefacts. An example of such a spectrum is given in *Figure 5.1*, which is a stacked plot where the slices of data incrementing in t_1 are drawn successively.

This 2D experiment is equivalent to the series of spin-decoupled spectra usually required to assign proton couplings and has the advantage of greater dispersion: peaks in two dimensions are less likely to be coincident than in one dimension. Stacked plot presentations of 2D data take a long time to draw and can be difficult to interpret if peaks are hidden behind each other. The

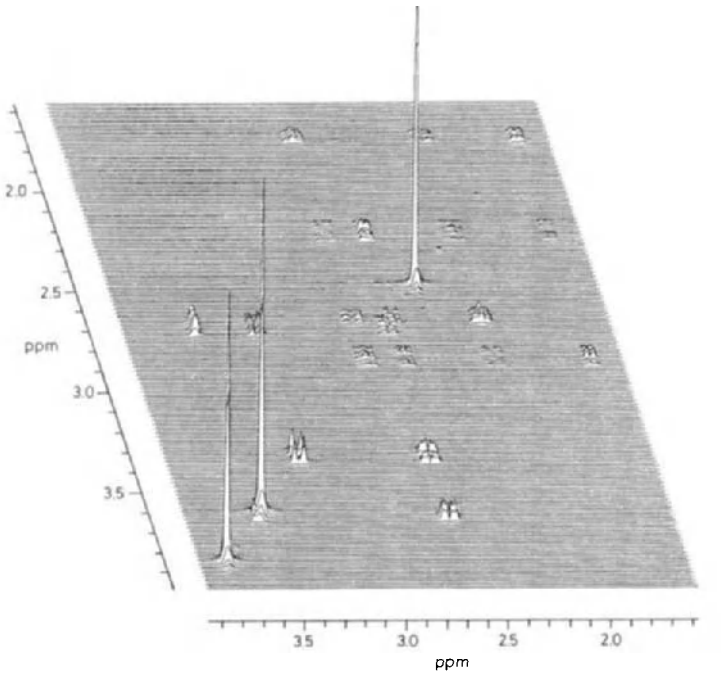


Figure 5.1. A stacked plot of the 2D COSY spectrum of thebaine in $CDCl_3$ at 400 MHz. The aliphatic region only is shown. The original spectrum had a frequency width of 2100 Hz in f_1 and f_2 and, after zero-filling once in f_1, a final matrix size 1024 × 512 which gave a resolution of 4.1 Hz per point in each direction. A sinebell window function was applied and the spectrum has been symmetrized. 128 scans were accumulated for each slice.

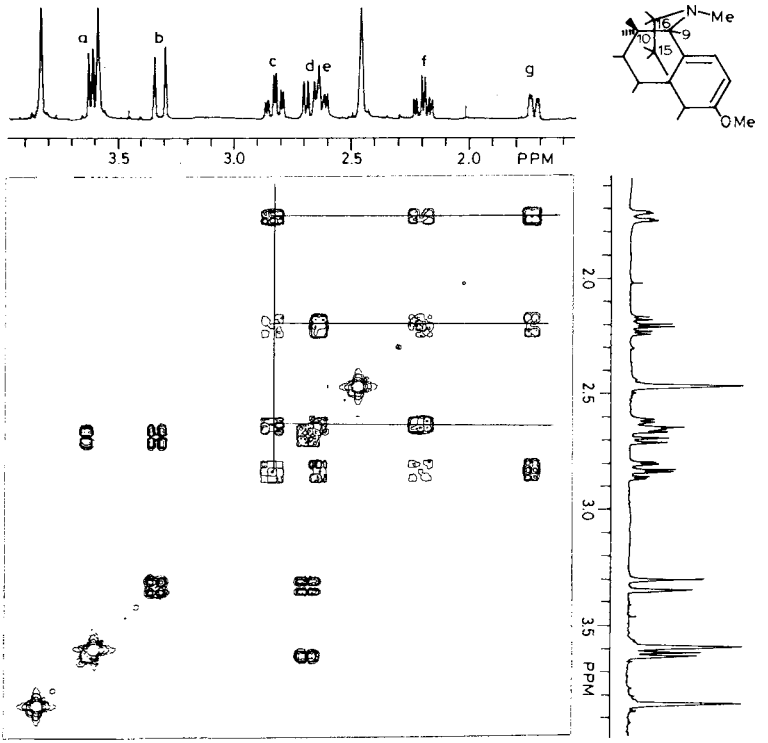


Figure 5.2. A contour plot for the same region of the 2D COSY spectrum of thebaine as shown in Figure 5.1. *This spectrum allows interconnections to be established between all groups of coupled signals (see partial formula), for example a (9-H) to d(10 α-H), b(10β-H) to d(10α-H), d to a and b; c(axial 16-H) to g(equatorial 15-H), f(axial 15-H) and e(equatorial 16-H). Connecting lines are drawn to show this last set. Resonances d and e overlap.*

preferred method of presentation is a contour plot in which peaks are represented by contours or thresholds as hills are on an ordnance survey map (*Figure 5.2*). An impression of the relative heights and sharpness of peaks is gained by examining the number of contours and how closely they are drawn together. The spectra are made easier to interpret by the use of different colours for successive contours.

The pulse sequence presented above gives a spectrum which reveals $^{1}H-^{1}H$ chemical shift correlations through spin-spin couplings and is the simplest of the 2D sequences. There is now a wide variety of experiments available, many

built in to the software available on commercial instruments, in which the series of pulses and time intervals have been tailored to give different types of spectral information. Two-dimensional experiments can be broadly classified into two groups: chemical shift correlation spectroscopy and *J*-resolved spectroscopy. Some examples of the different types of experiment will be given here and examples of the applications given later in the review. More detailed reviews of the techniques available can be found elsewhere [5, 17–20] and textbooks directed at the research chemist which discuss the application of multipulse sequences in one and two dimensions have recently been published [21–23].

CORRELATION SPECTROSCOPY

The Jeener experiment described above which gives proton–proton correlations through spin-spin coupling is known as COSY (from **CO**rrelation Spectroscop**Y**). When the second 90° pulse is replaced by a 45° pulse, the cross-peaks are simplified in a way that depends on the sign of the coupling constant. This may be useful for differentiating vicinal and geminal coupling constants in aliphatic systems (as discussed later, *Figure 5.41*). In addition, the diagonal peaks are also simplified so that correlations near the diagonal are more readily observed. The COSY experiment may be optimized by using additional fixed delays between pulses [24] to reveal long-range couplings which may be of value in structural analysis, for example, such interactions being stereospecific in rigid systems [25]. Pulse sequences which make use of multiple quantum transitions, which can be observed only indirectly, have been developed to simplify spectra. For example, the double quantum filtration (DQF) version of COSY, which is achieved by an extra 90° pulse at the end of the sequence, eliminates singlets such as those arising from water or methyl groups which can obscure peaks near them in the spectrum and thus improves the dynamic range [26].

A variety of heteronuclear shift correlation experiments have been developed for different pairs of nuclei, the ^{13}C-^{1}H shift correlation based on the DEPT pulse sequence being the most commonly used (an example is given later, *Figure 5.42*) [27]. The experiment can be optimized by appropriate adjustment of time intervals to give correlations due to long-range C–H couplings which are particularly useful to observe connectivities across non-protonated carbons. COLOC is a sequence designed specifically to reveal these small couplings [28]. Assignment of coupling pathways which remain ambiguous may be aided by relay experiments in which the magnetization from one nucleus is transferred or relayed through a second nucleus to a third,

thus giving correlations between two nuclei each coupled to a mutual one. This technique can be applied to homonuclear and heteronuclear experiments [29, 30].

Signals can also be correlated through Nuclear Overhauser Enhancement (NOESY) [31], which gives a spectrum similar to COSY with peaks due to both NOE and scalar coupling. NOESY spectra, therefore, must be interpreted in conjunction with a COSY spectrum. Careful choice of the pulse interval in which the NOE develops is necessary and may require several experiments to find the optimum.

J-RESOLVED SPECTROSCOPY

J-resolved spectroscopy is used to resolve overlapping multiplets by giving spectra which have chemical shift on one axis and scalar coupling on the other. The multiplets are in effect turned on a central axis through 90° so that a projection perpendicular to the chemical shift axis gives resolved apparent singlets and a projection perpendicular to the coupling axis gives the splitting pattern for each signal. Early development of 2D NMR was concentrated on this pulse sequence to simplify the analysis of the complex spectra of large molecules such as nucleic acids and proteins. Homonuclear and heteronuclear versions have been developed [32, 33].

BIOLOGICAL NMR

Perhaps the most dramatic developments have been in the field of biological NMR. The introduction of very high field NMR spectrometers has increased the resolution of complex spectra of biomolecules such as peptides, proteins and polysaccharides, while the application of 2D experiments has aided spectral assignment. Dynamic interactions between ligands and macromolecules have been investigated by NMR which can give kinetic data and information on structural aspects of binding. Examples are the interaction between substrates or inhibitors and enzymes, drugs and receptors, proteins, immunoglobulins or nucleic acids. Specific examples are discussed later in this review.

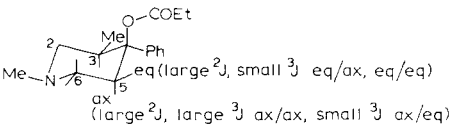
NMR has been used to investigate drug metabolism *in vitro* and *in vivo*. High-field FT spectrometers offer sufficient sensitivity for the measurement of some drugs and their metabolites, along with endogenous metabolites, in biological fluids such as plasma, serum and urine (see later section on Drug Metabolism). The advantage that NMR can offer over other techniques is the simultaneous quantification and identification of substances but sensitivity is the limiting factor in such analyses. Phosphorus metabolism has been studied

in vivo using specially designed 'surface coils' placed over the body [34]. *In vitro* metabolism can be followed in the NMR tube using cell suspensions or cell-free extracts [34]. Early work on the measurement of water in biological tissues has led to the development of imaging techniques which exploit the differential relaxation times of water in various body tissues (magnetic scanning) [35].

The examples which follow have been chosen to illustrate applications of these novel NMR techniques. In most sections, the approach has been to draw data from various classes of agent of interest to medicinal chemists rather than discuss each type of experiment in sequence. The review concludes with a short section on solid-state NMR.

OPIOIDS [35a]

400 MHz ^{1}H-NMR spectra of the isomeric prodines (alpha and beta) provide striking examples of the degree of resolution attainable by use of a very high frequency spectrometer. Early stereochemical arguments based on the 60 MHz spectra of these compounds were indirect and restricted to minor differences between the few resolvable signals (aromatic, 3-Me, ester $O\underline{CH_2}\underline{CH_3}$) [36]. In the higher frequency spectra, however, all resonances are resolved in the β-case and all but two (axial 5-H and $O\underline{CH_2}$ Me overlap) in the spectrum of α-prodine (*Figure 5.3* and (1)). Identification of the ring proton resonances is of particular importance since it provides direct evidence of the configurations and preferred conformations of these molecules. Thus, of the six resonances numbered (1–6) of this kind in the spectrum of α-prodine, 1, 2 and 5 must arise from the equatorial member of a CH_2 pair because they display only one large coupling (^2J) while 3 and 4 are typical of axial members with two large couplings (^2J and ax,ax ^3J); all lines are broadened by small couplings (resolved in some cases) due to vicinal interactions between axial and equatorial or a pair of equatorial protons (see (1)). Resonance 6 is due to the 3-H proton – removal of couplings to 3-methyl converts it into a double doublet with separations (large 12.3 Hz, small 4.2 Hz) which prove this proton to be axial and hence 3-Me must be equatorial (*Figure 5.3*, inset A). When the



(1)

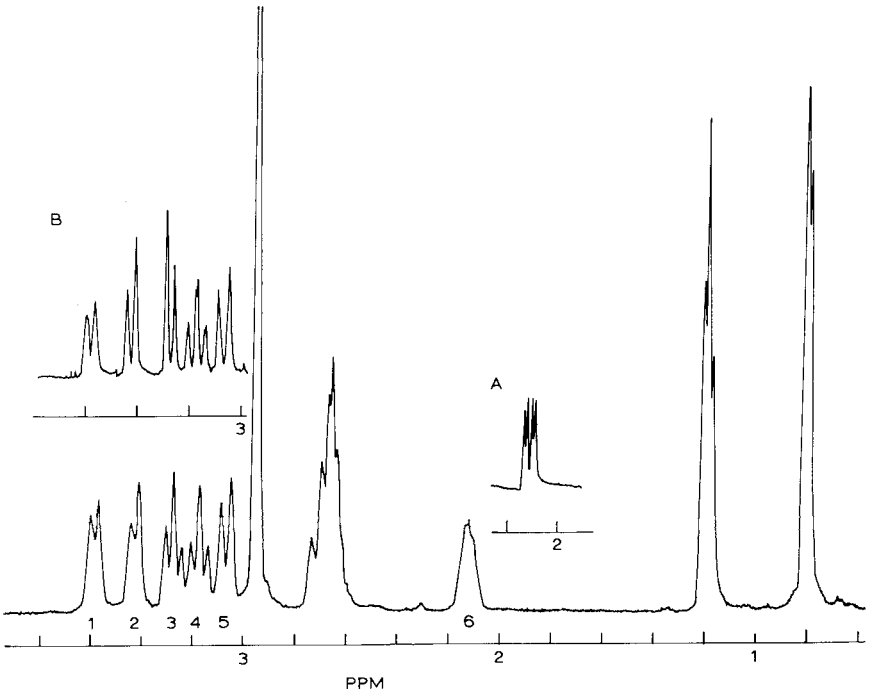


Figure 5.3. High-field region of the 400 MHz ^{1}H-NMR spectrum of α-prodine hydrochloride in D_2O. Inset A shows appearance of signal 6 (3-H) after irradiation of the 3-methyl doublet. Inset B shows appearance of signals 1–5 after irradiation of signal 6.

3-H resonance (6) is irradiated, the triplet (3) becomes a broad doublet (2) and is therefore assigned to axial 2-H, while the broad doublet (2) appears as a sharp doublet and must arise from equatorial 2-H (*Figure 5.3*, inset **B**). The assignments triplets (4) and doublet (1) to axial and equatorial 6-H, respectively, follow on chemical shift grounds (proximity to electronegative $^+$NH). The remaining unassigned doublet (5) must be due to equatorial 5-H, while the axial 5-H signal is obscured within the multiplet to low field of signal (6). The spectrum of β-prodine may be analysed similarly and assignments of both diastereoisomers are listed in *Table 5.1*. The data fully support equatorial 3-methyl (α) and axial 3-methyl (β) chairs as the preferred solute conformations in D_2O; differences between α,β 3-H and α,β axial 2-H resonances have particular significance in this respect (β axial 2-H is lower field than the α-signal because it is deshielded by axial 3-methyl) [37].

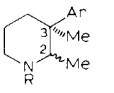
Spectra of certain isomeric 3-aryl-3-methylpiperidines are also well resolv-

Table 5.1. ^{1}H-NMR CHEMICAL SHIFTS OF α- AND β-PRODINE HYDROCHLORIDE IN D_2O[a] (SEE [1])[b]

Isomer	*2-H*		*3-H*		*5-H*		*6-H*		*3-Me*	*OCH_2*	*Me (ester)*	*N-Me*
	ax	*eq*	*ax*	*eq*	*ax*	*eq*	*ax*	*eq*				
α	3.28 brt (12.5)	3.43 brd (9.3)	2.13m[c]	–	~2.6 unres.	3.07 brd (16)	3.18 brt (13.5)	3.59 brd (11)	0.80 (7)	~2.6 unres.	1.19t (8)	2.95
β	3.74 dd (12.9, 3.6)	3.57 brd (13)	–	2.64[d] brq	~2.75 dt (15, 4.3)	~2.95 (~16)	3.22 dt (13.6, 2.9)	3.41 brd (13)	0.74 (7.6)	2.46 q (7.6)	1.04 t (7.6)	2.92

[a] In ppm from DSS, multiplet separations (Hz) in parentheses; abbreviations: d, doublet; t, triplet; q, quartet; br, broad.
[b] In betaprodine the 3-Me substituent takes the axial position at C-3.
[c] dd (12.3, 4.2 Hz) when 3-Me irradiated.
[d] br singlet when 3-Me irradiated.

ed under high field conditions. Compounds of this class have been shown to behave as opioid agonists and antagonists, dependent upon the nature of their N-substituent, for example (2) [38].



(2)

opioid antagonists

α(*t* 2, 3-di-Me)	1 X nalorphine
β(*c* 2, 3-di-Me)	2 X nalorphine

(Ar = *m*-HOC_6H_4, R = $CH_2CH{=}CH_2$)

The spectrum of the 3,5-dimethylpiperidine of *Figure 5.4* (shown to be the *trans* 3-Ar, 5-Me isomer, see below) is a typical example; assignments to ring proton signals (5 isolated, 2 overlap) are detailed in the legend [38]. A key assignment was identification of the 5-H resonance, shown to be the multiplet (f) because it lost couplings when the 5-methyl doublet was irradiated. This enabled a critical NOE difference experiment to be performed which differentiated the configuration shown in *Figure 5.4* from that in which the 3-substituents are reversed (that is, 3-Ar equatorial, 3-Me axial). When irradiation was applied at 1.9 ppm (signal f, axial 5-H), clear positive NOEs were observed for the *ortho*-aromatic, 5-methyl and equatorial 4-H protons, results clearly diagnostic of the axial 3-aryl arrangement (*Figure 5.5*). An isomeric form of this piperidine was isolated which must be the *cis*-3-Ar,5-Me form, making putative opioid ligands available with preferred axial or equatorial 3-Ar substituents. Isomers of this kind are, in fact, most rapidly characterized by ^{13}C-NMR spectroscopy. Since axial methyls in alicyclic six-membered rings are more subject to steric polarization effects than equatorial methyls (see p. 376), compounds with 3-methyl resonances in the range 23.2–25.8 ppm must have axial, 3-methyls and those in the range 30.5–31.8 ppm have equatorial 3-methyl groups [38].

Analysis of the ^{1}H-NMR spectrum of α-metazocine provides a useful entry to those of morphine and other 4,5-epoxymorphinan opioid ligands. All eight alicyclic ring proton resonances of α-metazocine may be resolved at 270 MHz even though some overlap of signals occurs (*Figure 5.6*). Signal 2 (a double doublet, dd) displays two small couplings (about 2.5 and 6 Hz) and may be assigned to 1-H, since this proton lacks a geminal partner and is only weakly coupled to the α- and β-8-H protons (dihedral angles are about 30° to the α- and 90° to the β-proton). The 6 Hz coupling of signal 2 is also apparent in the signal 3 dd, while the larger coupling of this resonance (16 Hz) is repeated in the doublet of signal 1, and must be a ^2J interaction, hence the assignments signal 1: β-8-H, signal 3: α-8-H may be made. The lower-field double triplet (5) must arise from axial 3-H, and its higher field counterpart (7, partially

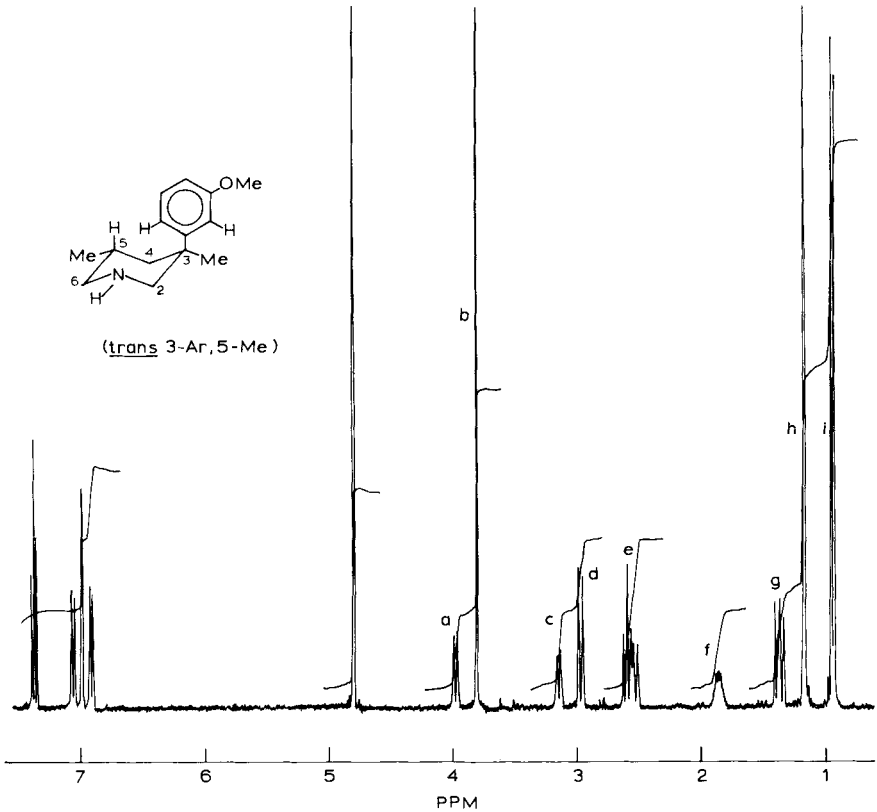


Figure 5.4. 400 MHz ^1H spectrum of the trans*-3-Ar, 5-Me compound hydrochloride shown above in D_2O. Assignments: (a) eq-2-H; (b) OMe; (c) eq-6-H; (d) ax-2-H; (e) ax-6-H, eq-4-H; (f) ax-5-H; (g) ax-4-H; (h) 3-Me; (i) 5-Me. The 5-H resonance was readily assigned (see text); the eq-2-H (a) was differentiated from the ax-2-H (d) resonance by its long-range coupling to eq-4-H (e, higher field part) that operates via a W pathway.*

masked) from axial 4-H. Assignments of the broad doublets 4 (overlaps N-Me) and 8 (overlaps 5-Me) to equatorial 3-H and 4-H respectively follow, while the 9-H signal (6, doublet of quartets, separations 3 and 7 Hz) overlaps signal 7.

The key to assignment of the more complex spectrum of codeine base (*Figure 5.7*, all signals resolved at 270 MHz in $CDCl_3$) is identification of the β-10-H resonance near 3.05 ppm (equivalent to β-8-H of α-metazocine) which is characterized by its sharp doublet of large separation (18 Hz). Its α-10-H

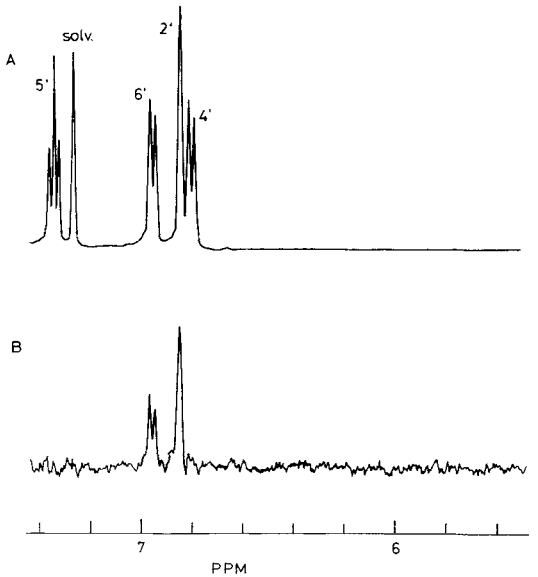


Figure 5.5. Aromatic region of the NOED 400 ^1H MHz spectrum of the trans-*3-Ar-5-Me compound 3-aryl-3,5-dimethylpiperidine hydrochloride in D_2O. (A) control with irradiation position remote from resonance signals and showing the presence of the complete aromatic signals (5'-H t, solvens s, 6'-d, 2'-s, 4'-d, low- to high-field order); (B) irradiation at 1.9 ppm (5-H) showing the presence of 2' (s) and 6' (d) aromatic signals only.*

partner gives rise to the double doublet near 2.3 ppm (separations 18 and 6 Hz), while the 4-line signal near 3.35 ppm (separations of 6 and 2.8 Hz) is assigned to 9-H which in turn connects to the narrow multiplet near 2.68 ppm (14-H) (cf. assignments of α,β-8-H, 1-H and 9-H of the α-metazocine spectrum). The vinylic protons 7-H and 8-H have characteristic chemical shifts and couplings while one (6-H) of the two protons linked to an oxygen atom is assigned to the broad multiplet near 4.2 ppm and 5-H to the double doublet at 4.9 ppm. Interconnections between the various protons are revealed by the COSY 45 plot (*Figure 5.8*) which establishes that the lower-field vinylic proton of ring C is coupled to 6-H, 5-H and 14-H (in addition to its vinylic partner) while the higher-field signal is coupled only to 6-H and 14-H. It is most reasonable, therefore, to assign the lower-field signal to 7-H and the higher to 8-H on the grounds of the greater proximity of 7-H to 5-H (expansion of the vinylic signals clearly reveals that the lower-field resonance is the more extensively coupled of the two, as shown in *Figure 5.7*). In the spectrum of 3,6-di-*O*-acetylmorphine (heroin) base, the 6-H resonance moves to low field

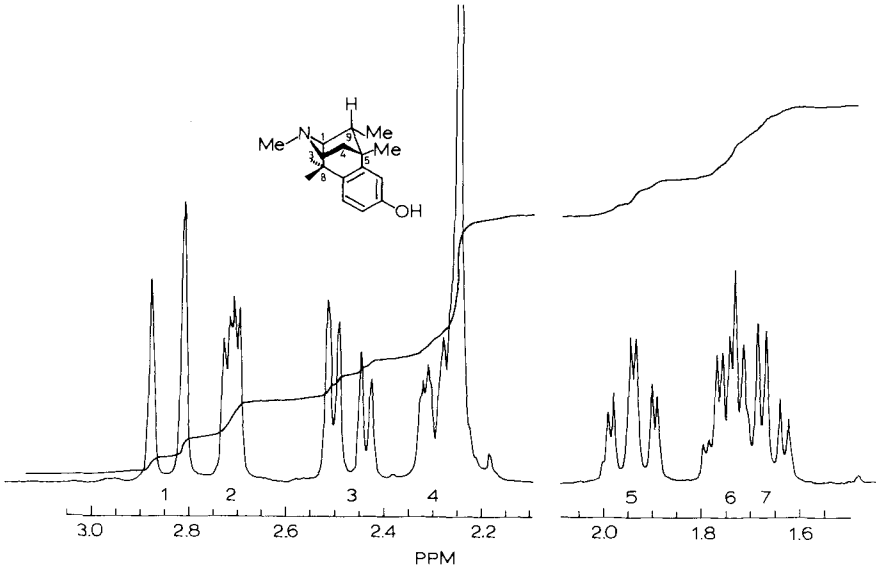


Figure 5.6. Part of the 270 MHz ^1H spectrum of α-metazocine in DMSO-d_6. Aromatic signals (6.4 – 6.9 ppm), eq-4-H (signal 8 of text) and 5-methyl (overlap near 1.2 ppm) and 9-methyl (d, 0.75 ppm) resonances are not shown.

of the 5-H resonance as a result of operation of the acylation shift [40] (see inset to *Figure 5.7*). The axial 15-H and 16-H double triplets and the equatorial 15-H and 16-H broad doublets may be assigned on chemical shift grounds (16-H lower field than 15-H signal because of proximity to nitrogen).

14-Hydroxy analogues of morphine such as oxymorphone and naloxone are readily identified by the absence of a 14-H resonance in their ^1H-NMR spectra, and the presence of a two-line rather than a four-line 9-H signal (doublet coupled only to α-10-H). Saturation of the 7,8-double bond is manifest in the absence of low-field vinylic signals and greater complexity of the spectral region to high field of 3 ppm due to addition of four extra signals, most of which are resolved. A slow but measurable H–D exchange takes place at the C-7 position of naloxone at neutral or acidic pH in D_2O with preferential deuteration of the 7-axial proton (intensities of dt near 2.9 ppm and d near 2.2 ppm diminish with time) [41, 42].

Perly, Pappalardo and Grassi [41, 43] have analysed the 600 MHz ^1H-NMR spectra of morphine, nalorphine and oxymorphone hydrochlorides and obtained evidence of molecular conformation from ^3J coupling magnitudes.

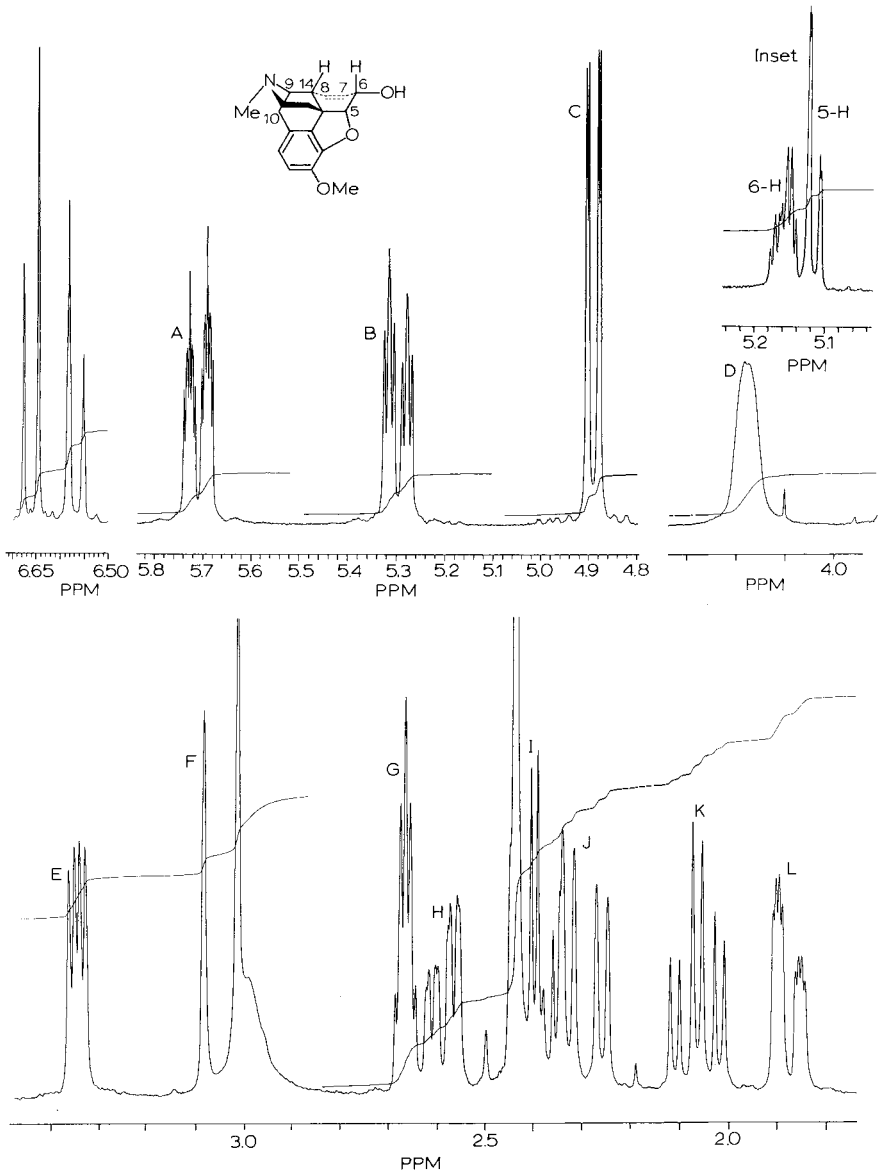


Figure 5.7. Part of the 270 MHz ^1H spectrum of codeine in $CDCl_3$. Assignments: A 7-H; B 8-H; C 5-H; D 6-H; E 9-H; F β-10-H; G 14-H; H/L eq 15-H, 16-H of piperidine ring; I/K ax 15-H; 16-H of piperidine ring; Jα-10-H. The inset shows the 5-H and 6-H resonances of diacetylmorphine (heroin).

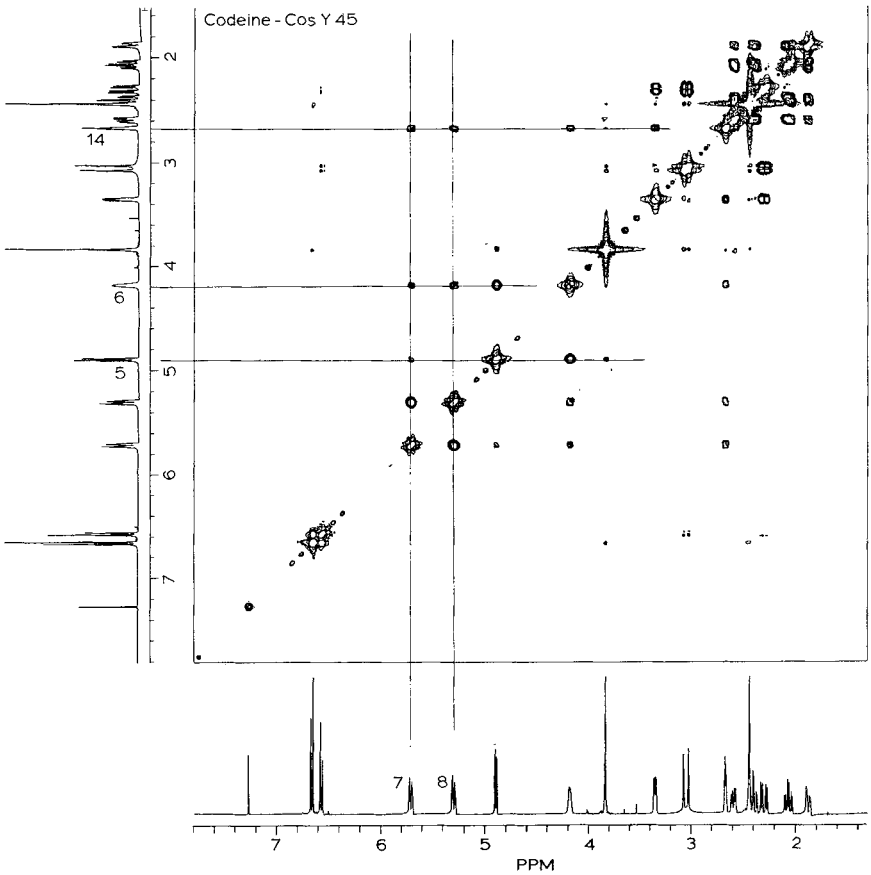


Figure 5.8 Proton correlated 400 MHz COSY 45 spectrum of codeine in $CDCl_3$. Interconnections between vinylic protons 7-H and 8-H with 5-H, 6-H and 14-H protons are shown by the horizontal and vertical grid lines.

Their results are summarized in *Figure 5.9*; similar conformations were proposed for the 14-hydroxy analogues naloxone and naltrexone [41].

The ^{1}H-NMR spectra of salts of morphine and its congeners are complicated by the existence of protonated epimers, evident from signal duplication and first reported in a 600 MHz study carried out at low pH to slow the interconversion rate (*Figure 5.10* and [44]). The usual pronounced preference of *N*-methyl for the equatorial conformation in piperidine derivatives is reduced in the fused-ring system of morphine by non-bonded interactions with the β-10-

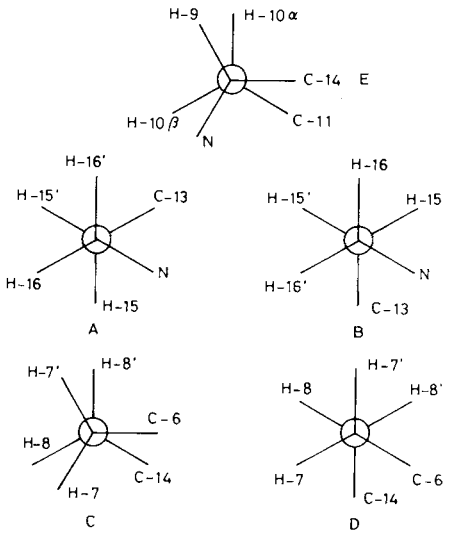


Figure 5.9. Newman projections for conformations about C-C bonds for hydrochloride salts of morphine (72 °C), nalorphine (57 °C) (A,B,E) and oxymorphone (23 °C) (A–E). Both conformations A and B are compatible with J_{exp} values. Calculation of the rotation angle for which $J_{calc} = J_{exp}$ in A gives an angle for a distorted-chair conformation of the piperidine ring. Conformation D agrees selectively with J_{exp} data for oxymorphone: this molecule will adopt a chair conformation of the ring C(5), C(6), C(7), C(8), C(13), C(14), corresponding to the staggered conformation D about the C(7)–C(8) bond. The J_{exp} value in all compounds fits the single possible conformation E allowed by this fixed molecular portion (an angle of ca. *30 ° was evaluated between 9-H and α-10-H axes in arrangement E).*

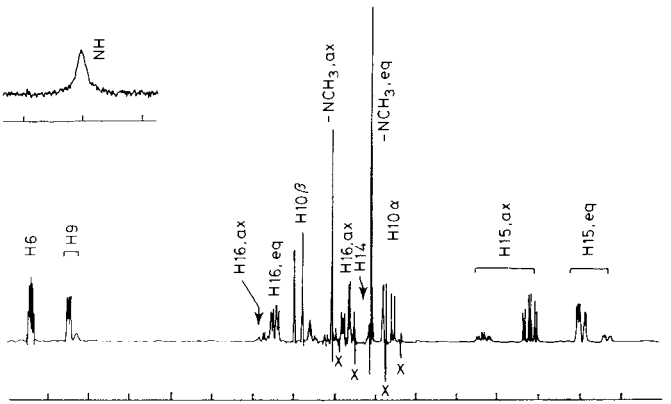


Figure 5.10. 600 MHz ^{1}H-NMR spectrum of morphine hydrochloride in D_2O, pD ≈ 1.5 (region upfield of the water signal). The major and minor components of the 15-H axial and 15-H equatorial signals are bracketed and the major and minor components of the two N*-methyl resonances are labelled.*

H proton, and an eq-NMe/ax-NMe ratio of about 5 is found for morphine and nalorphine salts based on integration of major and minor 9-H resonances. The eq-NMe epimer is more favoured in oxymorphone and naloxone (ratio near 24) due to destabilizing by ax-NMe-9-OH interactions and/or stabilization of the eq-NMe epimer by intramolecular hydrogen bonding of type $^{+}$NH...OH(C-9) [42].

Glasel and Borer [45] have investigated the mode of binding of opioid ligands at monoclonal antibody sites by an NMR method that may well be valuable when it becomes possible to study ligand interactions with the opioid receptor itself. Their procedure involves transfer NOE experiments (p. 389) which detect closely placed proton pairs and hence provide evidence of the binding conformation. The 500 MHz ^{1}H-NMR spectrum of naloxone in the presence of antibody showed general line broadening over that observed for free ligand in accord with rapid exchange between free and bound species. Thus, irradiation of the exchange process broadened lines in order to observe transfer NOE phenomena from the bound forms of the ligands was considered justified. No intramolecular NOEs were found in the case of free nalorphine solution. However, for a nalorphine monoclonal antibody (12 D4) solution, strong transfer took place between the 7-H proton and the terminal CH_2 of the *N*-allyl group. This result is explained if the binding conformation of nalorphine is one in which the *N*-allyl substituent is axial.

A comparative analysis of the 500 MHz ^{1}H-NMR spectra of morphine and its three *O*-acetyl derivatives has been published by Neville, Ekiel and Smith of the Drug Identifications Division of the Canadian Government, as an aid to the detection of 3- and 6-monoacetylmorphine in illicit heroin samples [46].

CARBON-13 NMR SPECTRA

A major consequence of the introduction of pulse (FT) NMR spectroscopy has been ready access to ^{13}C data – limited prior to 1970 by factors which render this magnetic nucleus relatively insensitive to continuous wave methods of recording NMR spectra (1% natural abundance, and low value of the nuclear magnetic moment compared with that of a proton). ^{13}C-NMR spectra are generally much simpler than corresponding ^{1}H spectra. When run under conditions where all couplings to protons are removed (by simultaneous wide-band irradiation of proton resonances), a ^{13}C-NMR spectrum consists of a series of sharp lines, each of which corresponds to the resonance of a nucleus (or nuclei) of specific magnetic environment. Further, since the chemical shift spread of ^{13}C nuclei (0–200 ppm) is about 20-times that of protons,

the probability of complete resolution of signals due to each environmental type is high. Hence, a count of the number of signals observed often tallies with the number of carbon atoms in different types of magnetic environments in a molecule and provides a rapid means of assessing the *purity* of a sample. For example, we recently employed a 20-year-old sample of 3-methyl-1-phenethyl-4-piperidone as a synthetic intermediate. Its spectrum (*Figure 5.11*) displayed the anticipated 12 signals (no more and no less) and could be used confidently without further purification.

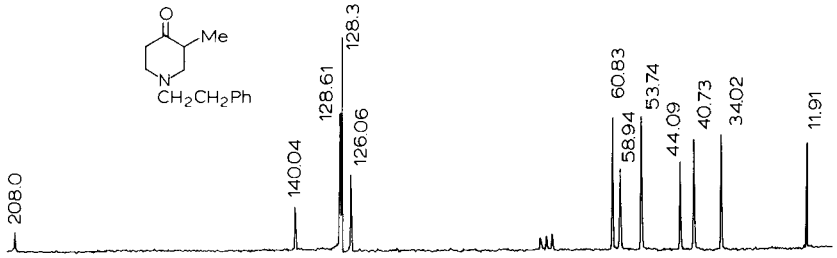


Figure 5.11. ^{13}*C-NMR proton-decoupled spectrum of 3-methyl-1-phenethyl-4-piperidone in* $CDCl_3$ *at 22.5 MHz.*

Signals in excess of those required generally denote the presence of impurities – they may, however, give evidence of the isomeric nature of the sample. A 4-piperidone again provides a relevant example. The spectrum (*Figure 5.12a*) of 1,2,6-trimethyl-4-piperidone prepared by a route stereospecific for the *cis* isomer gave evidence of the isomeric purity of the product [47]; that of material obtained by a non-stereospecific route (decarboxylation of the intermediate (3)), however, displayed five additional signals – evidence that it was a *cis-trans* mixture of the two ketones (*Figure 5.12b*). If desired, separation of the two isomers could have been monitored readily by ^{13}C-NMR spectroscopy, since isomeric signals were well resolved. The relative configurations of the two isomers are confirmed by the higher field 2(6) CH and 2(6) Me resonances of the *trans* form (see below).

The resolution of ^{13}C-NMR spectra is even greater when super-high-field magnets are employed, but because of the extensive chemical shift range of ^{13}C, the enhancement is less dramatic than is the case for proton spectra.

Assignment of ^{13}C resonances and their use in structural elucidation is based, as in ^{1}H-NMR, upon chemical shift correlations and spin-spin coupling interactions (valuable collections of chemical shift data are included in text by Stothers [48] and Breitmaier and Voelter [48a]). In proton-decoupled

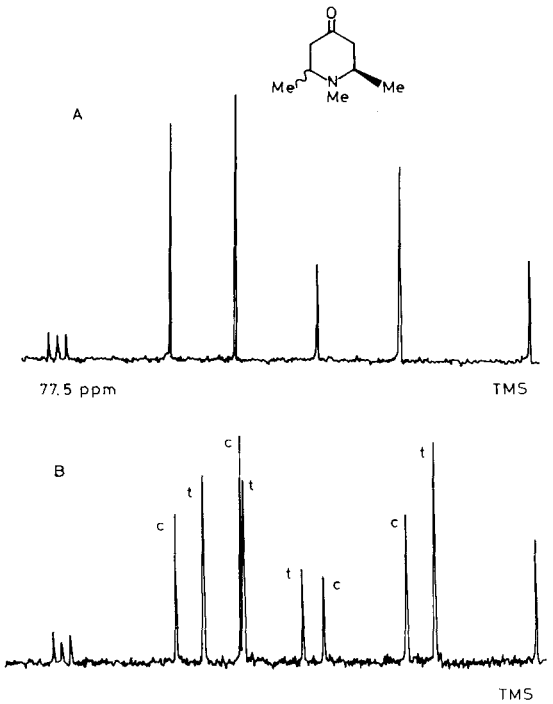


Figure 5.12. ^{13}*C-NMR proton-decoupled spectrum of (A)* cis-*2,6-dimethyl*-N-*methyl-4-piperidone, and (B)* cis/trans-*2,6-dimethyl*-N-*methyl-4-piperidone in* $CDCl_3$ *at 22.5 MHz (carbonyl resonances not shown).*

^{13}C-NMR spectra, coupling interactions are sacrificed for the sake of spectral simplicity and resolution (fully coupled spectra are complex because of substantial ^{13}C-^{1}H coupling over one, two and three bonds, see below), but techniques are available that yield such information without undue loss of resolution. Until recently the off-resonance procedure has been the most commonly employed. In this, the frequency position of the irradiation used to decouple the protons is moved somewhat away from optimum so that a small proportion of the ^{13}C-^{1}H couplings (normally of about 150 Hz magnitude) is retained, whereby singlets (Cq), doublets (CH), triplets (CH_2) and quartets (CH_3) may be recognized provided signal overlap is not extensive. The spectrum of the 3-arylpiperidine of *Figure 5.13*, a compound related to agents with opioid ligand activity [37] provides a typical example. The nature of the carbons giving rise to the single lines of *Figure 5.13* (upper, decoupled spectrum) is revealed by their appearance in the off-resonance variant (*Figure 5.13*

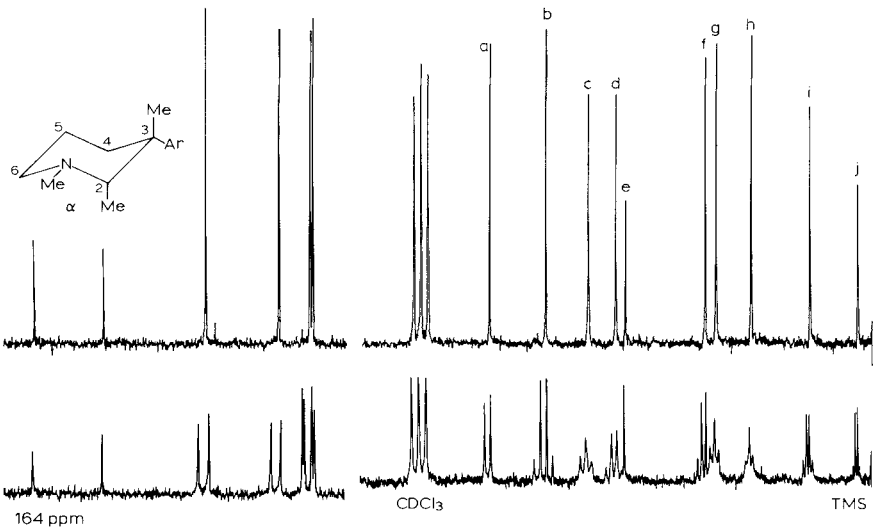


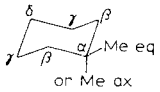
Figure 5.13. ^{13}*C-NMR spectra of a* cis *(2-Me/3-Ar) 1,2,3-trimethyl-3-arylpiperidine hydrochloride (Ar =* m*-methoxyphenyl) in* $CDCl_3$. Upper: *proton-decoupled spectrum;* lower: *off-resonance spectrum.*

lower). Multiplicities of the aromatic signals are as anticipated (2 Cq, 4 CH). The high field singlet (e) must arise from C-3 and the doublet (a) from C-2. The three triplet signals (c, g and h) are due to carbons 4, 5 and 6 and may be assigned specifically on chemical shift grounds. Quartets identify the four methyl signals (b, d, f and i); from chemical shift arguments, (b) is assigned to OMe, (d) to NMe and (f, i) to the 2,3-methyls. Spectral comparisons with the corresponding derivative isomeric about C-2 allow configurational and conformational conclusions to be drawn, and illustrate the value of ^{13}C-NMR in elucidating the molecular geometry. A key principle is the steric polarization influence of a bulky substituent such as methyl in saturated 6-membered rings when axially disposed [49]. γ-Placed carbons suffer this effect and, in consequence, their resonance positions are upfield by several parts per million of positions seen in the absence of axial methyls or when the methyl group is equatorially placed. Full details of methyl substituent parameters for substituted cyclohexanes are given in *Table 5.2*.

Application of these parameters to solving the stereochemistry of some 2,3-dimethyl-4-arylpiperidines is now outlined [38, 50] in respect of chemical shift data on the triad $\alpha-(4)$, $\beta-(4)$ and a 2-demethylanalogue (*Table 5.3*).

The C-4 shifts provide the stereochemical key, and show that 2-methyl must be axial in the α-isomer (cf. also the C-6 shifts). The C-2 and C-3 shifts

Table 5.2. METHYL SUBSTITUENT SHIELDING PARAMETERS IN CYCLOHEXANES [49]



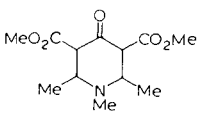
Substituent	*Parameters (ppm)*[a]			
	α	β	γ	δ
Equatorial-Me	+ 5.6 ± 0.2	+ 8.9 ± 0.1	0.0 ± 0.6	− 0.3 ± 0.2
Axial-Me	+ 1.1 ± 0.4	+ 5.2 ± 0.3	− 5.4 ± 0.2	− 0.1 ± 0.3
Geminal (Me_2)	− 3.4 ± 0.6	− 1.2 ± 0.4		
Vicinal (Me_2) dieq	− 2.3 ± 0.3			
ax/eq	− 3.1 ± 0.6			

[a] +, deshielding (low-field shift).
−, shielding (high-field shift).

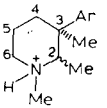
Table 5.3. ^{13}C-NMR CHEMICAL SHIFTS OF 1,3-DIMETHYL-3-(*m*-METHOXYPHENYL)PIPERIDINE HYDROCHLORIDE AND OF THE CORRESPONDING α- and β-2-METHYL ANALOGUES IN $CDCl_3$[a] [38]

R	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*3-Me*	*2-Me*
H	60.1	36.7	33.3	19.1	52.8	23.6	–
α-Me	64.9	40.9	25.2	19.1	47.6	27.0	8.7
β-Me	67.6	42.3	38.7	20.3	56.4	17.5	12.8

[a] In ppm from TMS.



(3)



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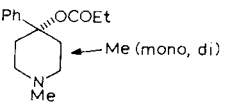


(5)

demonstrate that a methyl substituent (axial or equatorial) deshields both the carbon to which it is directly attached (α) and that one removed (β). For all three isomers, ^{1}H-NOE experiments gave evidence that 3-methyl has a preferred axial position (see below). The α-2-methyl shift has an unusually high-field position typical of 2-methyl axial to $^+$NH in piperidines and related

heterocycles [51]. In the β-isomer the axial 3-methyl resonance is at higher field than that of the same carbon of the 2-demethyl analogue as a result of γ-shielding by the equatorial 2-methyl substituent; in the α-isomer the signal moves downfield because of the deshielding influence of the axial 2-methyl group in this isomer [52].

Stereochemical assignments to isomers derived by methylation of the pipe-

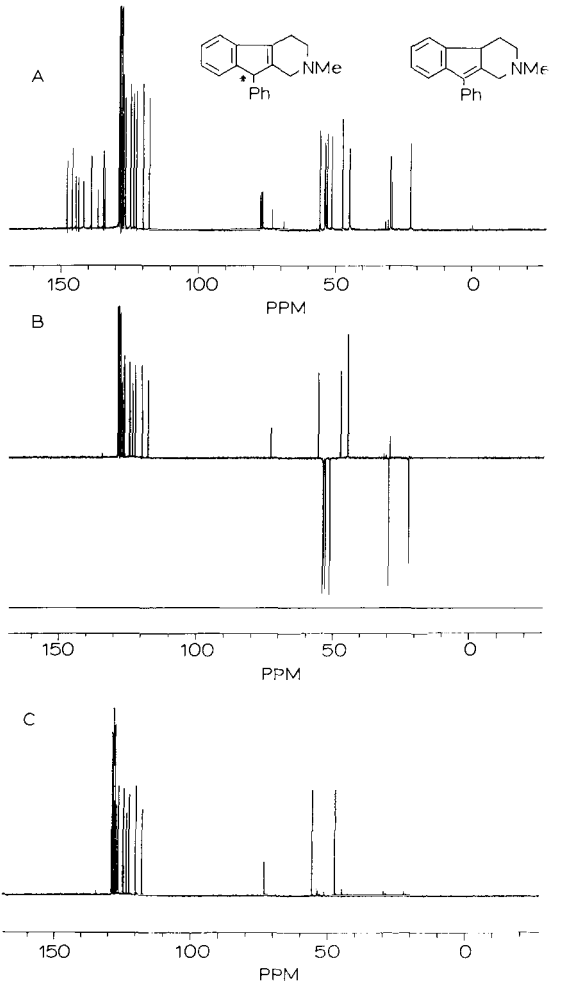


Figure 5.14. ^{13}C NMR spectra of phenindamine base in $CDCl_3$ at 67.5 MHz. (A) Normal proton-decoupled spectrum; (B) 135 DEPT (CH,CH_3 + ive, CH_2 − ive Cq absent); (C) 90 DEPT (CH only).

ridine ring of the reversed ester of pethidine (5), for example, 2,5-dimethyl derivatives such as promedol and 2,3- and 2,6-dimethyl analogues, have also been facilitated by similar analyses of ^{13}C-NMR chemical shift data [53]. Recently, Whitesell and Minton [53a] have drawn attention to the fact that two vicinal hydrogens in an anti relationship contribute to a downfield shift for each of the carbon atoms involved.

The interpretation of off-resonance spectra becomes difficult if not impossible in cases where resonances are closely placed and extensive overlap of multiplets occurs. In such situations, more recent pulse sequences such as INEPT and DEPT remove uncertainties of this kind. Thus the DEPT procedure provides (i) a spectrum in which methine (CH) and methyl carbons give positive lines (all singlets), methylene (CH_2) give negative signals (Cq signals are absent), and (ii) one showing CH (positive) resonances only; from this set together with a fully decoupled spectrum, all carbon types may be assigned without ambiguity.

DEPT spectra of the antihistamine phenindamine in base form are shown as an example (*Figure 5.14*); the data reveal the base to be a mixture of the two positional isomers shown in the *Figure 5.14*. The six methylene carbons (three for each compound), two methines and *N*-methyl resonances (44.8, 44.7 ppm, seen resolved at high expansion) are clearly identified from amongst the many closely placed lines of the normal decoupled spectrum (a), while all the Cq lines (2 × 5) may be found in the low-field region of the spectrum [54].

Examples of the use of fully coupled ^{13}C spectra are given in the antibiotics section (p. 400).

SMALL PEPTIDES

Low-resolution ^{1}H-NMR spectra of small peptides are difficult to analyse because of extensive signal overlap. Excellent resolution is possible, however, under high frequency operating conditions, as demonstrated during the late 1970s for enkephalins – naturally occurring pentapeptides which bind to opioid receptors [55]. The case of the synthetic analogue (6), in which the Gly^2 residue of Met (or Leu)-enkephalin has been replaced by

Tyr-DNle-Gly-Phe-D(or L)NleS

(6)

DNle and the carboxylate terminal residue-5 by the D or L sulphonic acid analogue of Nle (NleS), is presented here [56]. The spectrum (*Figure 5.15*) was run in DMSO-d_6, a solvent which permitted the identification of the peptide NH signals and NH,α-CH couplings. Rapid N–H exchange rates in

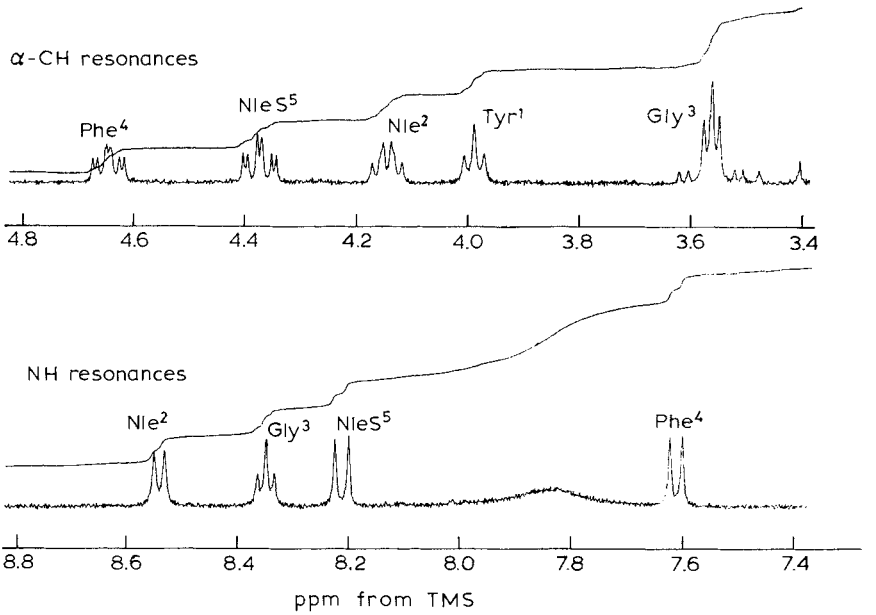


Figure 5.15. Part of the 400 MHz ^1H spectrum of the Tyr-amino-terminal peptide Tyr-DNle-Gly-Phe-LNleS in DMSO-d_6 at 23 °C.

peptides usually preclude studies in water. However, NH resonances may be observed when the pH is lowered below neutral to minimize base-catalysed NH exchange. Thus, Mosberg and Schiller [57] achieved this for some cyclic enkephalin analogues by examining them in a 90% H_2O/10% D_2O mixture adjusted to pH 3.5 with CD_3CO_2D. The drawback to this procedure is that conformational evidence so derived may not reflect conformations at physiological (near neutral) pH. When a substantial amount of water is present in the NMR solvent saturation of the water resonance or use of special pulse sequences must be carried out to minimize the HDO resonance (see p. 421) [58, 59]. The magnitudes of NH, α-CH, together with those of α-CH, β-CH couplings, and the relative sensitivities of NH shifts to rises in temperature provide evidence of intramolecular hydrogen bonding and hence evidence of peptide conformation (see below). The dipeptide Ac-LPhe-DNleS served as a reference compound for assignment of the pentapeptides; its spectrum (*Figure 5.16*) is almost fully resolved at 400 MHz in DMSO-d_6. A key to the assignments is provided by the results of spin-decoupling the α-proton signal at 4.41 ppm, which causes the higher-field NH doublet to collapse and the

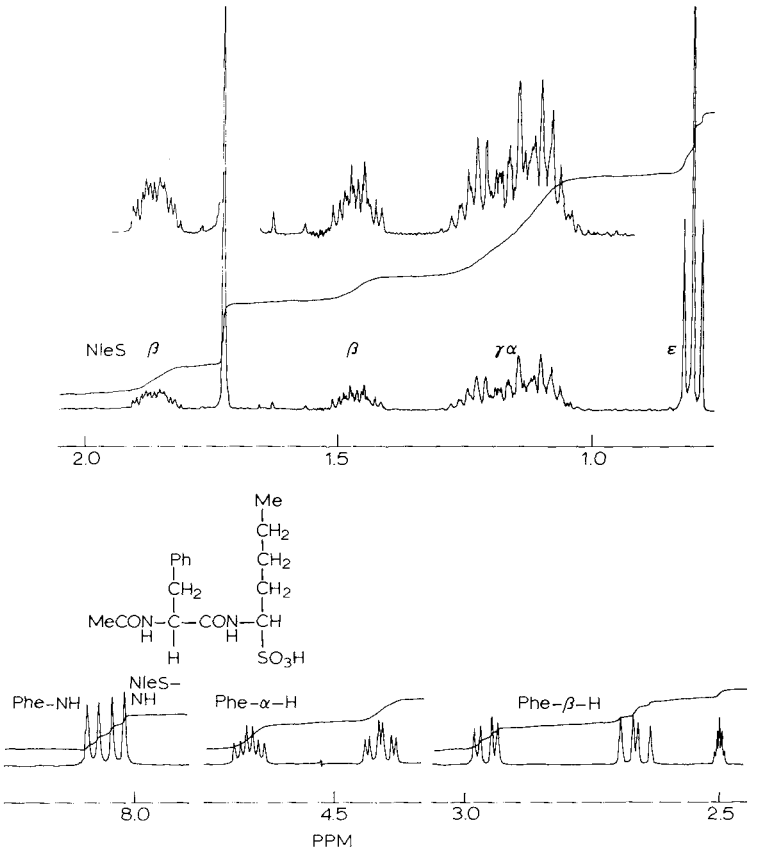


Figure 5.16. Part of the 400 MHz ^{1}H-NMR spectrum of the dipeptide Ac-LPhe-DNleS in DMSO-d_6 (multiplet at 2.5 ppm is due to the solvent).

one-proton multiplets centred at 1.86 and 1.46 (assigned to the β-protons of the DNleS residue) to lose some couplings. The 8-line CH_2 signal between 2.5 and 3 ppm is diagnostic of methylene adjacent to a chiral methine carbon (CH_2*CH, * denotes chiral carbon) and its chemical shift range is characteristic of phenylalanine residues. The spectrum of the D^2L^5 pentapeptide (*Figure 5.15*) is likewise well resolved and many trivial assignments are possible such as the Gly^3 NH (triplet), the Tyr^1 α-CH (triplet little changed after D_2O addition). The Tyr^1 and Phe^4 β-CH_2 signals can be assigned on the basis of typical shifts and separations in spectra of related peptides [59]. All four NH signals are resolved together with those of the phenolic and $^+NH_3$

protons. Of the remaining α-CH signals, that at 4.65 ppm is due to Phe^4 (linked to the Phe β-CH_2 signals by spin decoupling, cf. the Phe α-CH_2 assignment of the model dipeptide), while those at 4.15 and 4.38 ppm must be due to the Nle^2 and Nle^5 residues. Specific assignments are possible and are based on spin-decoupling effects on the one proton multiplet centred near 1.9 ppm which is considered to be one of the β-protons of NleS (compare data on the model dipeptide); the latter signal becomes narrower and better resolved after irradiation of the α-CH signal at 4.38 ppm and is little changed by irradiation at 4.15 ppm. With the α-CH resonances identified, the assignment of the NH signals likewise follows from the results of further spin decoupling experiments. Plots of the NH chemical shifts are linear over the range 25–100 °C and the rank order of NH temperature coefficients (δ, ppm $°C^{-1}$) is $Phe^4 < Nle^2 < Gly^3 < NlesS^5$. Since the Phe^4 NH proton signal of the D^2L^5 peptide is significantly less affected by a rise in temperature than the other amide protons, the assumption is made that it is involved in an intramolecular hydrogen bond [60]. Its partner must be the carbonyl of Tyr^1, that is, that of the amino acid three residues removed from Phe, because bonding to C= O of residues 2 or 3 is not sterically feasible. A conformation which permits such a bond requires a β-turn about the Nle^2 C-N bond as shown in *Figure 5.17*.

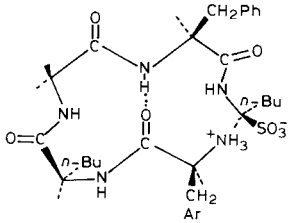


Figure 5.17. Diagrammatic representation of one conformation of the Tyr-amino-terminal peptide Tyr-DNle-Gly-Phe-LNleS, with C-H hydrogens omitted for clarity.

This model accommodates observed NH, α-CH coupling magnitudes for all residues within the limits of the appropriate plots of ^3J against dihedral angle [61]. The spectrum of the D^2D^5 pentapeptide provides no evidence of intramolecular interaction and hence the peptide is less likely to adopt a conformation of the type shown in *Figure 5.17*. In contrast, NMR studies of enkephalins terminated with Leu, Nle or Met carboxylate residues all give evidence of 2–5 intramolecular hydrogen bonding between the carboxylate terminal NH and carbonyl oxygen of residue 2 [55, 62]. Since these peptides included diastereoisomeric pairs that differ pharmacologically (for example [DAla^2, DLeu^5]- and [DAla^2, LLeu^5]enkephalins), it is improbable that their solute

geometry in DMSO-d_6 has any significant bearing upon their interactions with opioid receptors.

DIHYDROFOLATE REDUCTASE

Studies of the binding of antifolate drugs to the enzyme dihydrofolate reductase (DHFR) carried out by Feeney and Roberts and their colleagues at the National Institute for Medical Research (Mill Hill) provide examples of a diverse range of NMR applications.

Fundamental to such work is assignment of as many of the ^1H signals of the protein as possible, and this has now been achieved for some 20% of the residues of DHFR. The enzyme from *L. casei* (M_r 18,500, 162 residues) [63] contains 16 valine and 14 leucine residues, and assignment of their 60 methyl resonances requires comparison of spectra of the normal enzyme with that in which [γ-$^2H_6$]valine has been incorporated (both examined as complexes with an inhibitor, methotrexate or trimethoprim). *Figure 5.18* shows the high-field region of the 500 MHz ^1H spectrum of complexes of the normal (a), and deuterated (b) enzyme [64]. Resonances missing from (b) must arise from valine methyl groups, while the difference spectrum (c) contains only the 32 methyl resonances from the 16 valine residues present in the enzyme. Only two of these resonances are resolved, namely, V_B (Val^{61}) and V_A (Val^{110} or Val^{115}, subsequent assignment), as a result of being shifted to high field by magnetic shielding influences of nearby aromatic rings; the bulk of the valine methyl resonances lie in the envelope between -2.5 and -3.4 ppm. The COSY spectrum of the normal enzyme complex allows the two methyl groups of each valine-leucine residue to be identified because these produce pairs of cross peaks as a result of their connection to a common ($C_\beta H$ or $C_\gamma H$) ω_1 frequency in the 2D matrix (*Figure 5.19*) [64]. Thus in *Figure 5.19*, two methyl resonances at -3.90 and -3.38 ppm give cross peaks with a common ω_1 frequency of -2.2 ppm and they thus arise from the same valine or leucine residue (valine in fact, see below), but in crowded regions of the spectrum it is difficult to pair up the two methyl resonances with certainty. COSY spectra obtained from the [γ-$^2H_6$]valine enzyme (24 h run required because of low concentration available) allow identification of the leucine methyl pairs while missing pairs must derive from the valine residues (*Figure 5.19B*). Any ambiguities that remain (for example, due to similarity of C–H chemical shifts) may be resolved by application of a homonuclear relayed coherence transfer (RELAY) experiment [65]. In this procedure, cross peaks are observed not only between pairs of protons that are scalar coupled (that is, via bonding

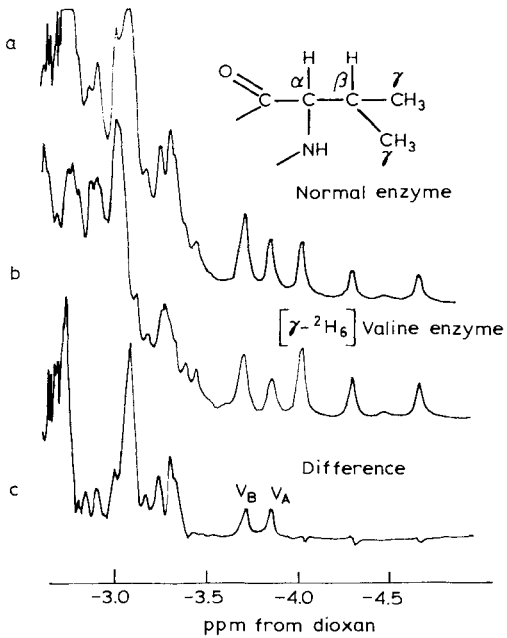
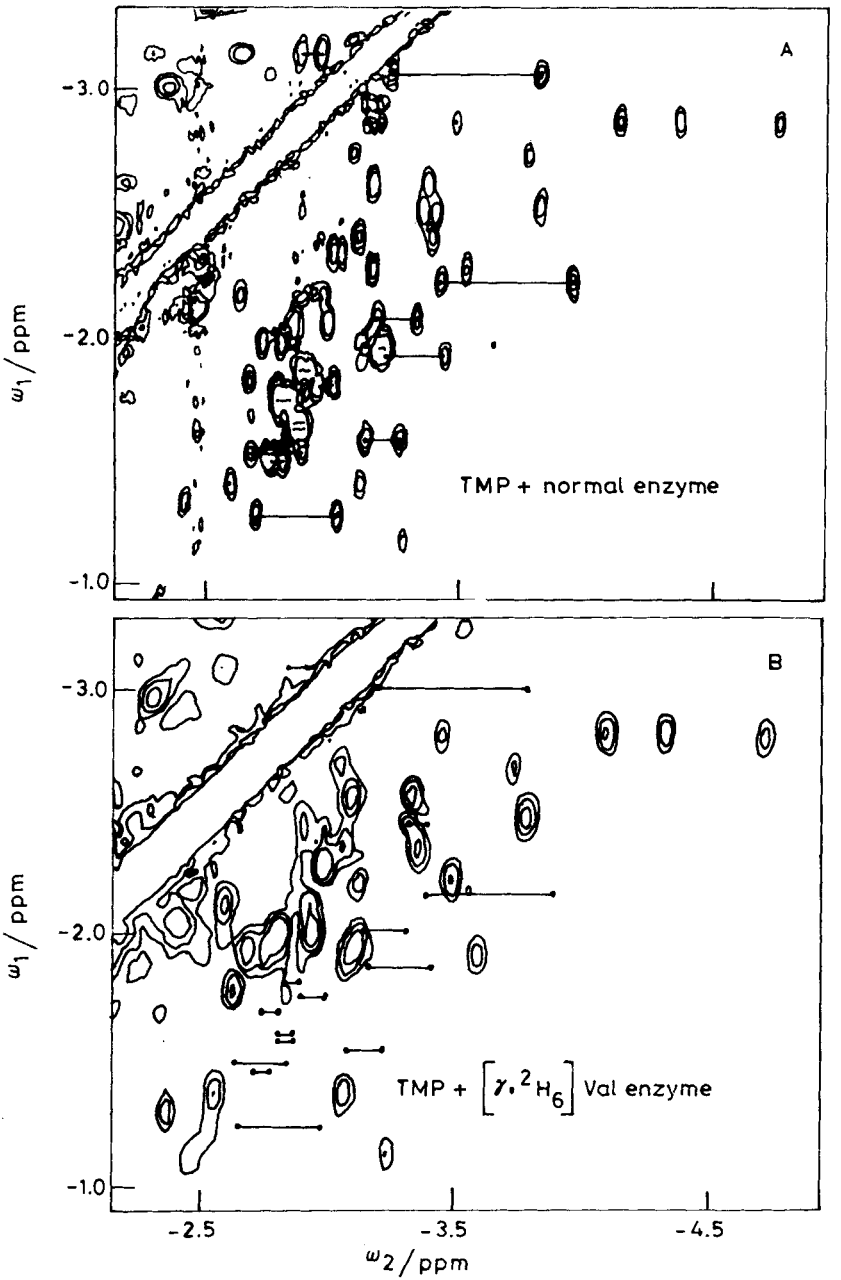


Figure 5.18. High-field region of the 500 MHz ^{1}H-NMR spectra of (a) the dihydrofolate reductase-trimethoprim complex and (b) the corresponding complex of the [γ-$^2H_6$]valine-containing enzyme. (c) is the difference spectrum (a)–(b). Structural formula shows carbon designations of the valine residues.

electrons) but also between two protons that each have a scalar coupling interaction with a third nucleus. Thus, assignment of two $C_\gamma H_3 - C_\beta H$ cross peaks (ω_1 – 2.39; ω_2 –3.94, – 3.38 ppm) is confirmed by the appearance of γ/γ and γ/α cross peaks (*Figure 5.20*). The RELAY experiment also allows a valine spin system to be traced from $C_\alpha H_3$ through $C_\beta H$ to $C_\gamma H$, an operation normally denied by the spectral complexity of a protein of this size.

This work represents a first stage only and subsequent assignment to individual residues in the protein sequence depends on NOE experiments and correlations with related X-ray structures, and has led to the solution of about 20% of the residues [66]. Further investigation of the high-field valine methyl

Figure 5.19. The high-field region of the 500 MHz ^{1}H 2D COSY spectra of (A) the dihydrofolate reductase-trimethoprim complex and (B) the corresponding complex of the [γ-$^2H_6$]valine-containing enzyme. The pairs of $C_\gamma H_3$-$C_\beta H$ cross-peaks from valine residues identified by comparison of these two spectra (see text) are connected by horizontal liines in (A); these lines are also included in (B), the dots at either end showing the positions of the valine cross-peaks present in (A) but missing in (B). ▶


A
-3.0
-2.0
-1.0
ω_1 / ppm
TMP + normal enzyme
B
-3.0
-2.0
-1.0
ω_1 / ppm
TMP + [γ-$^2H_6$] Val enzyme
-2.5
-3.5
-4.5
ω_2 / ppm

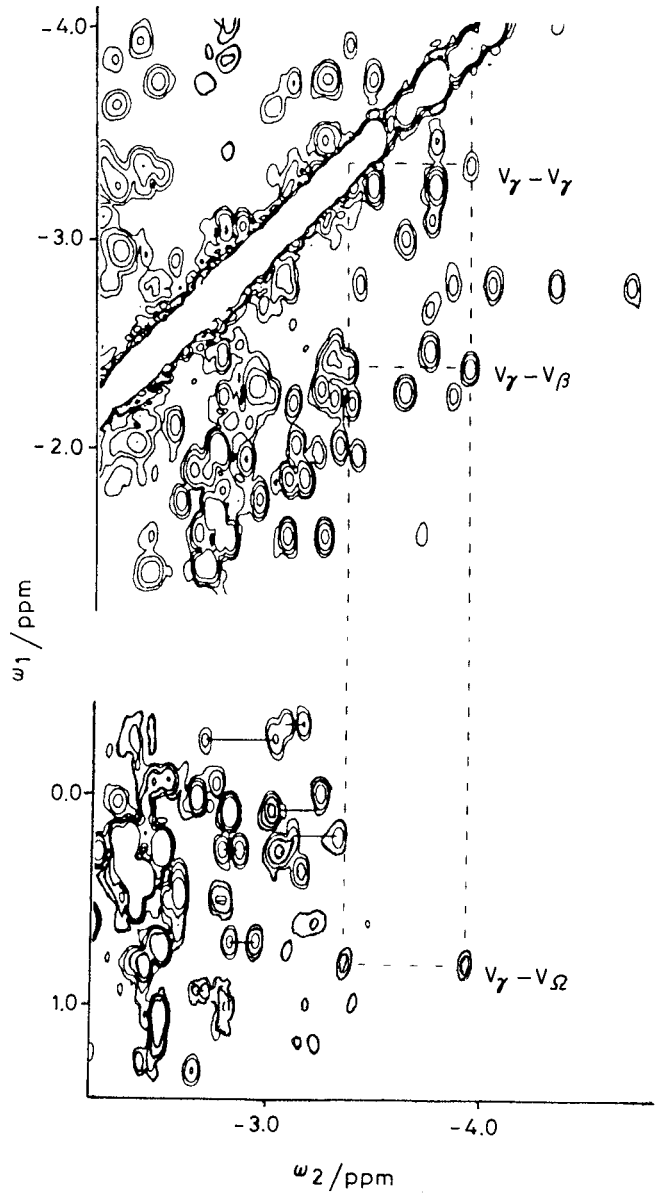


Figure 5.20. The high-field region of the 500 MHz ^1H 2D RELAY spectrum of the dihydrofolate reductase-trimethoprim-NADPH complex. The cross-peaks defining the spin system of valine V_A (Val^{61}) are connected by dashed lines; for details, see text. In the lower part of the figure, the pairs of valine $C_\gamma H_3$-$C_\beta H$ RELAY cross-peaks are connected by horizontal lines.

resonances (V_A and V_B, *Figure 5.18*) illustrates this point. The V_A methyl resonance (− 3.81 ppm) and its partner at − 3.36 ppm (linked by a spin-echo decoupling experiment and by 2D methods already described) are high field relative to methyl resonances of valine in simple peptides (about − 2.75 ppm from dioxan) due presumably to the shielding influence of nearby aromatic rings (ring-current shifts). Irradiation at − 3.81 ppm produced several NOE effects in the aromatic region of the spectrum which were too complex to analyse. However, use of deuterated protein in which all aromatic protons except the *ortho* (2′,6′) protons of tyrosine (Tyr) were replaced by ^2H allowed progress to be made. Five resonances for the *ortho* protons of the five Tyr residues may be seen in the spectrum of this enzyme (*Figure 5.21*). Irradiation at − 3.81 ppm (V_A) produced clear NOE effects at 3.33 and 3.18 ppm, so the valine methyl must be close in space (< 4.5 Å) to the aromatic protons of two Tyr residues. Inspection of the crystal structure showed that only Val^{61} is appropriate to this requirement – it is close to both Tyr^{46} and Tyr^{68}. Assignment of V_A and its associated methyl and H_β resonances, and Y_A and Y_C (*Figure 5.21*) to Tyr^{46} and Tyr^{68} is then possible. Additional NOE experiments involving irradiation of the *ortho* protons resonance of Phe^{49} allow assignment of Y_A to Tyr^{46} and Y_C to Tyr^{68}. In a definitive paper [66], the Mill Hill group

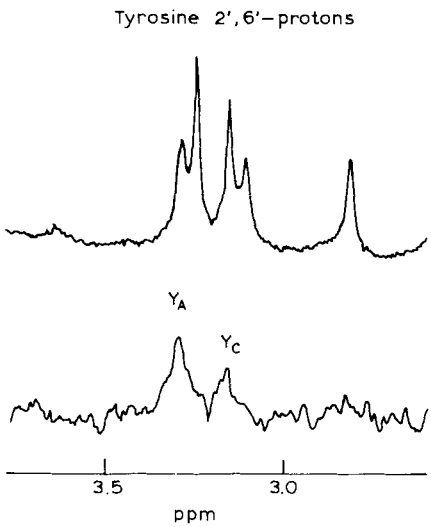


Figure 5.21. Upper: Aromatic region of 500 MHz ^1H-NMR spectrum of the complex between 2,4-diaminopyrimidine and selectively deuterated dihydrofolate reductase, in which the only aromatic protons remaining were the 2′6′-protons of the five tyrosine residues. Lower: Difference spectrum showing the NOE effects observed on irradiating resonance V_A in this sample.

report assignment of 32 of the 162 residues of the enzyme from *L. casei* in the form of its complex with (a) methotrexate, (b) methotrexate and $NADP^+$, and (c) methotrexate and NADPH chiefly based on procedures discussed above. Comparison of assigned resonances in the spectra of the three complexes provide evidence of structural difference between them. Fifteen of the assigned resonances differed by less than 0.05 ppm and since many of these arise from residues which lie in the β-sheet core and associated loops of the molecule, the constancy of their chemical shifts demonstrates that the binding of the coenzyme to the enzyme-inhibitor complex in solution does not produce significant changes in conformation of this region of the protein molecule. Chemical shift changes observed for the rest of the molecule provided evidence of coenzyme-induced conformational changes in the loop comprising residues 13 to 23, and one of the α-helices (residues 42 to 49).

Several kinds of information may be derived from *ligand* signals [67]. The ionization state of trimethoprim may be determined by studies with material labelled with ^{13}C at C-2 (*Figure 5.22*) to enhance signal intensity [68]. ^{13}C resonances due to both free and bound drug are observed in the spectrum of

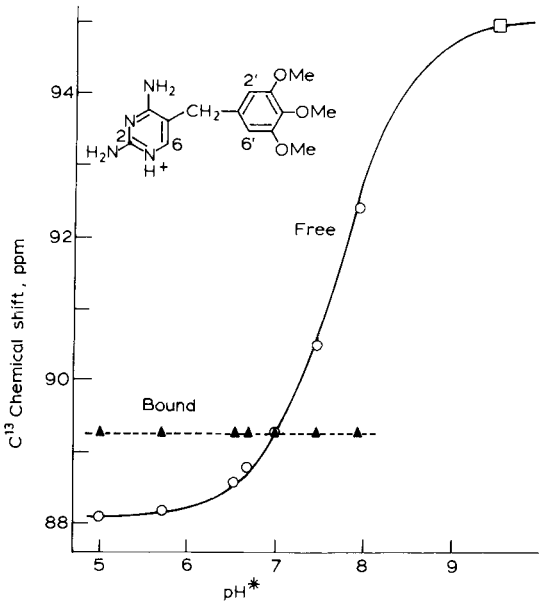


Figure 5.22. The pH titration curves of the ^{13}C chemical shifts of [2-^{13}C]trimethoprim (○) free (▲) in its complex with dihydrofolate reductase. The solid line is calculated for a pK 7.70 and a shift difference between protonated and unprotonated forms of 7.09 ppm.

a mixture of the enzyme and 2 molar equivalents of labelled material. The fact that the 2-carbon chemical shift in bound trimethoprim is much closer to that of the protonated molecule (about 88 ppm) than that of the base (about 94 ppm) suggests that N-1 is protonated in bound ligand. Further, the fact that the C-2 resonance (bound) does not alter over the pH range 5–8 shows that the pK_a of N-1 protonation has increased by at least 2 units on binding (pK_a of the free drug is 7.7 by NMR titration). These findings have been confirmed by ^{15}N-NMR using ^{15}N-N-1 labelled material in an experiment with an INEPT pulse sequence which relies on polarization transfer via ^{15}N scalar coupling with a directly attached proton (the ^{15}N chemical shift corresponded with the value for unbound protonated material) [69].

Conformational changes of ligands on binding may also be established. Interaction of trimethoprim with DHFR represents a case of an exchange equilibrium which is slow on the NMR scale, and which therefore gives rise to distinct resonances for free and bound ligand. In practice, signals due to bound ligand are often difficult to identify because of their broad nature and superposition upon complex protein resonances. The technique of transfer of saturation [70] may be used to overcome this problem. Spectra of *Figure 5.23* show the aromatic region of selectively deuterated DHFR in the presence of 2.7 molar equivalents of trimethoprim at 45 °C. The 2,6-proton resonances of the five Tyr residues and the two aromatic resonances of free trimethoprim (6,2′,6′, see Figure 5.22) are clearly observed in (a), but no bound ligand resonances are apparent. In transfer of saturation experiments, irradiation of a bound resonance should be relayed to the corresponding free signal as a result of the exchange process provided the relaxation rate is no greater than that of exchange (herein lies a means of measuring exchange rates). Thus, variation of the intensity of the 2′,6′-protons and 6-proton resonances of free trimethoprim as a function of irradiation frequency allows identification of resonances corresponding to the bound ligand (*Figure 5.24*) [71]. *Figure 5.24b* shows the results of a reverse transfer of saturation experiment in which the free 6-H resonance of free ligand is irradiated. Spectrum (c) of Figure *5.24*, shows the difference between (b) and (a) when irradiation is well removed from the aromatic region, and it reveals a signal at 2.76 ppm corresponding to a decrease in intensity of the bound 6-H resonance. In addition, it shows a signal at the position of the 2′,6′-H resonance of free trimethoprim which has also decreased in intensity on irradiation of the 6-H resonance. This result (not seen in the absence of the enzyme) is taken as evidence that 6-H and 2′-H (or 6′-H) are sufficiently close in space in *bound* trimethoprim for there to be a negative nuclear Overhauser effect on the 2′,6′-H signal on saturation of the 6-H resonance. This effect is then transmitted to the *free* species by

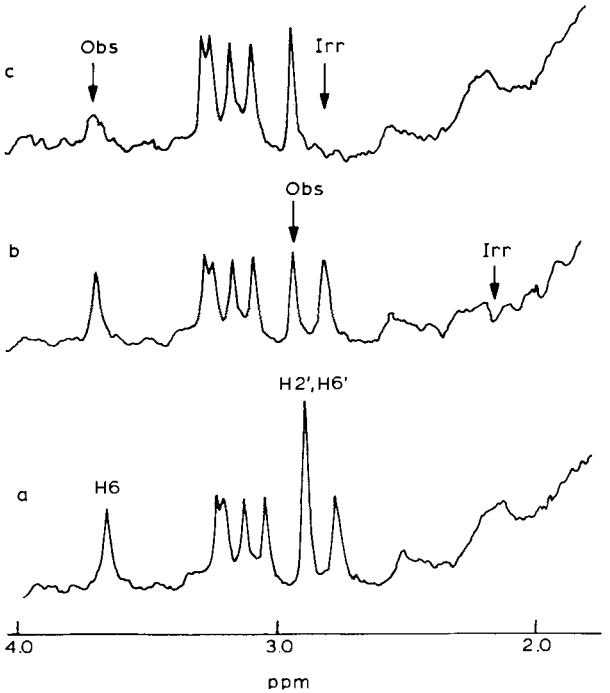
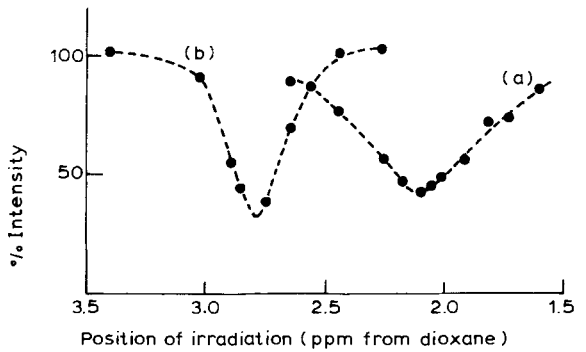


Figure 5.23. Upper: Aromatic region of the 270-MHz ^1H spectrum of selectively deuterated dihydrofolate reductase from L. casei *in the presence of 2.7 molar equiv. of trimethoprim at 45 °C. (a) Without irradiation; (b) with irradiation at 2.11 ppm; and (c) with irradiation at 2.76 ppm. The positions of irradiation and of those signals showing intensity changes are marked.*



Lower: Variation in intensity of the resonances of (a) the 2',6' protons and (b) the 6 proton of free trimethoprim as a function of irradiation frequency in a solution containing 1 mM selectively deuterated dihydrofolate reductase from L. casei *and 2.7 molar equiv. of trimethoprim.*

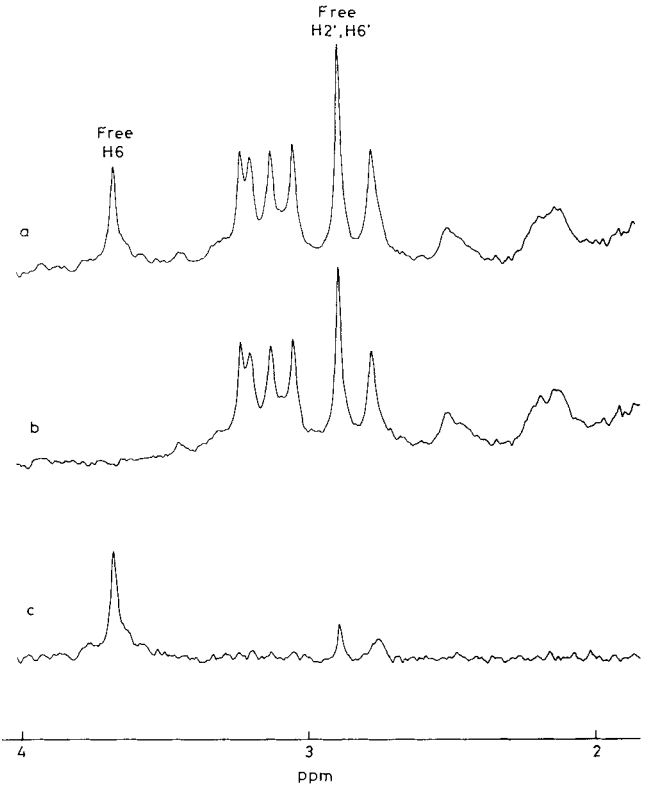


Figure 5.24. Aromatic region of the 270MHz ^1H spectrum of selectively deuterated L. casei *dihydrofolate reductase in the presence of 2.7 molar equiv. of trimethoprim (a) without irradiation, (b) with irradiation at the resonance frequency of the 6 proton of free trimethoprim, and (c) the difference between parts a and b.*

exchange [72] in a time comparable with or less than the spin-lattice relaxation time.

It is possible to use the transfer of saturation experiment to determine the dissociation rate constant of the complex by measuring the intensities of a resonance of free trimethoprim as a function of the length of time for which the corresponding bound resonance is irradiated (*Figure 5.25*) [71]. Measurement of free and bound ligand resonances establish that 6-H and one of the 2′,6′-protons of trimethoprim are shielded on binding. Examination of a range of conformations of trimethoprim show that the orders of shielding could not be accounted for solely by mutual (internal) ring-current effects, and it was

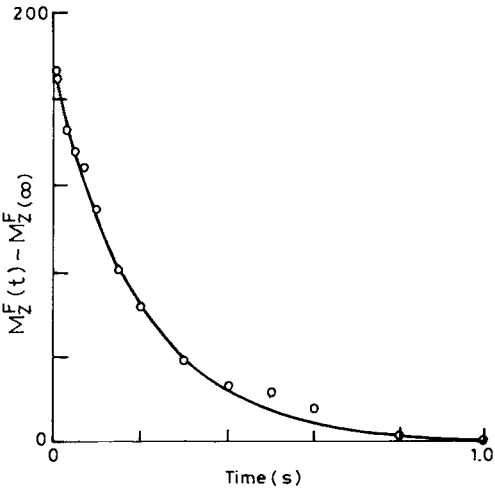


Figure 5.25. Change in magnetization, $M_z^F(t)-M_z^F(\infty)$, of the 6-H resonance of free trimethoprim as a function of the time, t, for which the bound resonance was irradiated. The curve is the best least-squares fit to the data, calculated by using the parameters in the table.

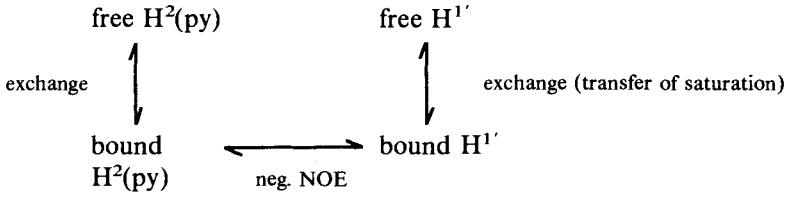
Dissociation rate constants, spin lifetimes, and relaxation times in the complexes of trimethoprim with dihydrofolate reductase at 45 °C.

Enzyme	Observed signal	τ_{1F} (s)	T_{1F} (s)	k_{off} (s^{-1})
L. casei	*H2′,H6′*	*0.22*	*0.72*	*5.2*
	H6	*0.20*	*1.22*	*6.8*
E. coli *(form II)*	*H2′,H6′*	*0.12*	*0.15*	*6.7*

considered likely that neighbouring amino-acid residues of the enzyme contribute to the chemical shift of the trimethoprim protons. Two ligand conformations based on internal ring-current effects and those due to the Phe-30 residue of the enzyme were advanced after study of the crystallographic structure of the *L. casei* enzyme methotrexate-NADPH complex. In both conformations, the 6-H and 2′,6′-protons were close enough to account for observation of an NOE effect between them (see above).

Transferred NOE (TNOE) methods in which NOE effects between nuclei in a ligand bound to an enzyme are transferred to the more easily detected nuclei in excessive free ligand as a result of the exchange of ligand molecules between the bound and free states, have also provided information about the

glycosidic bond conformations in complexes of $NADP^+$ and thio$NADP^+$ in *L. casei* DHFR [73]. Thus, selective irradiation of the $H^{1'}$ proton of the free coenzyme (see *Figure 5.26*) leads to a *decrease* in the intensity of the free H^2(py) proton signal due to the TNOE effect, while the H^6(py) proton is little affected. (Reasons for the phenomenon of negative NOEs have been presented [74].) The mechanism is depicted as follows:



This result requires close proximity of the $H^{1'}$ and H^2(py) protons in the bound state of the ligand, and establishes that the conformation of $NADP^+$ bound to DHFR is of the *anti* type, as shown in *Figure 5.26*. This is confirmed by observation of a substantial TNOE on the H^6(py) proton resonance after irradiating the frequencies of the $H^{5'}$ signals. The fact that at 20 °C, TNOE effects at H^2(py) of almost equal magnitude are obtained when the irradiation is centred on the $H^{1'}$ (free) and $H^{1'}$ (bound) resonances respectively, shows that the rate of exchange of the coenzyme between the two states must be faster than the relaxation rate of the $H^{1'}$ proton in the bound state. At 3 °C, when the dissociation rate is slower, irradiation at the $H^{1'}$ free position has a distinctly smaller effect on the intensity of the H^2(py) (free signal) than irradiation at the $H^{1'}$ (bound) resonance. The same methods show that thio$NADP^+$ binds with a distribution of *syn:anti* conformations very similar to that observed in nicotinamide mononucleotides in free solution (about 50:50). In contrast, both coenzymes had very similar *anti* conformations about their adenine glycosidic bonds when bound to the enzyme.

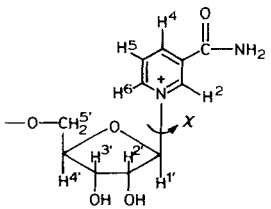
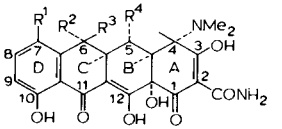


Figure 5.26. Partial structure of $NADP^+$ showing the nicotinamide glycosidic bond conformation.

ANTIBIOTICS

TETRACYCLINES

High-frequency ^{1}H-NMR spectra of tetracycline antibiotics allow resolution of many ring proton signals which fall within a 1.6–3.2 ppm envelope in 60 and 100 MHz spectra [75], for example, protons attached to C-5 and C-6 (see (7)). Such data, while improving the qualitative value of tetracycline spectra, provide direct evidence of the stereochemistry and preferred solute conformation of tetracycline derivatives gained from knowledge of proton coupling interactions within the 4,4a,5,5a,6-carbon system of (7).



	R^1	R^2	R^3	R^4	name
(7a)	H	Me	OH	H	tetracycline
(7b)	Cl	Me	OH	H	chlortetracycline
(7c†)	Cl	Me	OH	H	4-epichlortetracycline
(7d)	H	Me	H	OH	doxycycline
(7e)	H	H	Me	OH	6-epidoxycycline

†β-NMe configuration at C-4.

Proton resonances are generally well resolved in spectra recorded in DMSO-d_6 (an example is shown in *Figure 5.27*) [76] except for the 4a and 5a signals, which fall close to the *N*-methyl resonance. In many spectra, the *N*-methyl resonances are characteristically broad, a phenomenon which is probably the result of non-equivalence of the two *N*-methyl groups in the protonated salts. Assignments present little problem and may be substantiated by spin-decoupling experiments. The lowest-field non-aromatic signal is 4-H (adjacent to electronegative $^+NMe_2H$) which appears as a broad singlet. Of the two closely placed 4a-H and 5a-H multiplets near 3 ppm, that to lower field is assigned to 5a-H, since it does not include the small coupling displayed by the 4-H signal. The 5-H methylene signal near 1.7 ppm (see *Figure 5.27* inset) of derivatives lacking a C-5 substituent which displays three large couplings (resulting in an apparent quartet) is assigned to the pseudo-axial β-proton, and that near 2.2 ppm (showing one large and two small couplings) to the pseudo-equatorial α-proton (see *Figure 5.28*). These signals are absent in spectra of 5-hydroxy derivatives and replaced by a lower-field

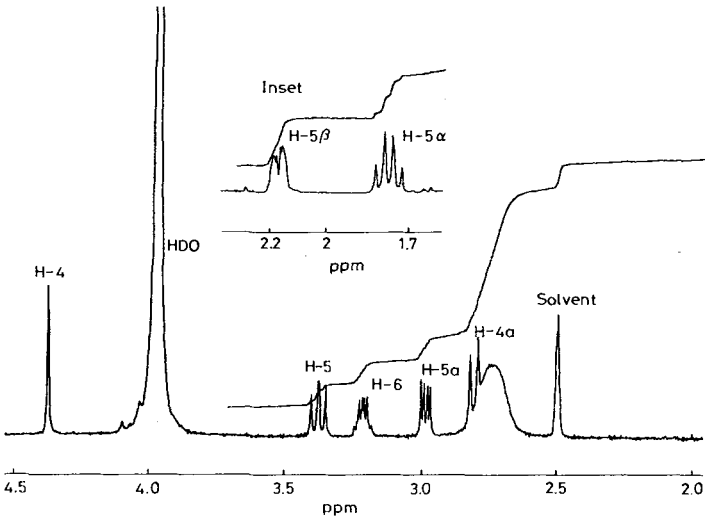


Figure 5.27. Part of the 400 MHz ^{1}H-NMR spectrum of 6-epidoxycycline (7e) hydrochloride in DMSO-d_6 plus D_2O. The broad signal near the H-4a resonance is due to the NMe_2 protons. Inset shows the H-5β and H-5α signals of tetracycline hydrochloride, typical of the 5-deoxy derivatives.

one-proton resonance (for example, dd 3.8 ppm for oxytetracycline). In doxycycline (7d) and its 6-epimer (*Figure 5.27*), the two additional methine proton resonances (5-H and 6-H) are readily distinguished by their coupling interactions (6-H is coupled to 6-Me and 5a-H, while 5-H is coupled to the single protons 4a-H and 5a-H only) [76].

Magnitudes of the coupling constants for protons attached to carbons 5a, 5, 4a and 4 provide evidence for the geometry of rings A and B of tetracycline derivative as solutes in DMSO-d_6. The results are in general agreement with conformations of the type established for hydrochlorides of tetracycline,

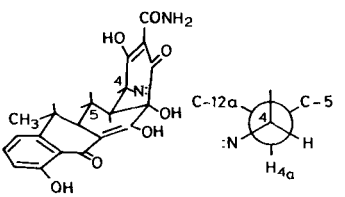


Figure 5.28. Conformation of hydrochloride of tetracycline derivatives; Newman diagram depicts view down C-4–C-4a bond.

chlortetracycline and oxytetracycline in the solid state [77]. In this arrangement (*Figure 5.28*), ring A is a half-chair aligned approximately at right angles to the plane of the B-D rings, with (12a-OH)-(12a-C)-(4a-C)-(4a-H) dihedral angles of about 60°. The small value of ^3J(4,4a) found in all derivatives is a key fact in support of this conformation, as are the large couplings established between pseudo-axial 5-H and 4a-H and 5a-H, respectively. Examination of Dreiding models shows that most other ring A,B conformations (all of greater flexibility than that of *Figure 5.28*) involve changes in dihedral angles that do not correlate with observed coupling constants.

NMR data for 4-epichlortetracycline (7c) require a conformation with a small dihedral angle linking 4-H and 4a-H (^3J(4,4a) 3.8 Hz) and one in which the dimethylamino substituent is in a highly hindered environment to allow for its its especially broad resonance, and an arrangement as shown in *Figure 5.29* is probable in this case [76].

Coupling constant magnitudes for doxycycline (7d) and its 6-epimer (7e) (both HCl salts) are consistent with solute conformations of the type shown in *Figure 5.28*. The 5-H resonance, which displays two large ^3J couplings confirms the α-5-OH configuration of the two compounds, while the ^3J(5a,6) couplings (13Hz for doxycycline and 4Hz for its epimer) establish that doxycy-

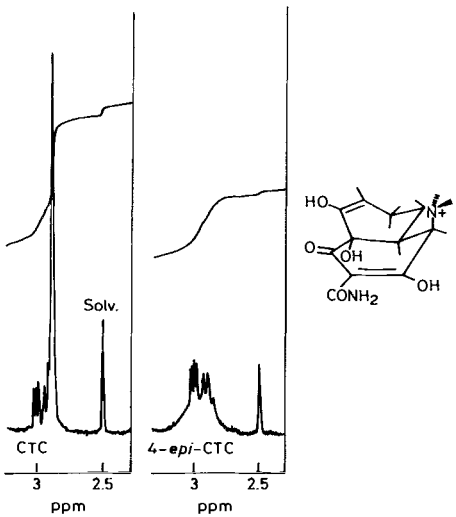


Figure 5.29. 3 ppm region of the 400 MHz ^{1}H-NMR spectra of hydrochlorides of chlorotetracycline (CTC) and 4-epi-CTC in DMSO-d_6 showing NMe_2, 4a-H and 5a-H signals. A conformation of 4-epi-CTC (partial structure) which places the dimethylamino group in a highly hindered environment is shown alongside.

cline is the α-6-methyl derivative as previously assumed on the basis of chemical evidence [79]. The configuration of 6-demethylchlortetracycline is likewise established as β-6-OH (pseudo-axial) by the fact that its 6-H resonance at 5 ppm forms a narrow doublet of separation (2.7 Hz) typical of a small 5a-H/6-H dihedral angle.

Coupling magnitudes for oxytetracycline base (dihydrate) in pyridine-d_5 are quite different from those seen in the spectrum of the hydrochloride salt, a fact which indicates that the base and protonated base conformations differ in a radical manner. The most striking difference is between the 4-H signals: 4.8 ppm, narrow doublet (few Hz separation) in the salt, 4.1 ppm wide doublet (sep. 10 Hz), in the base, indicative of a large (or very small) 4-H/4a-H dihedral angle in the case of the base. NMR features of 5,12a-diacetyloxytetracycline base are similar to those of the parent compound as solute in pyridine-d_5 (unpublished results): 4-H doublet at 4.06 ppm, separation 12 Hz. The solid-state conformation of the diacetate has been established by X-ray crystallography [78] and proves to be one in which ring A is twisted about carbons 4-4a-12a-1 of *Figure 5.28* into a half-boat format (*Figure 5.30*). This conformation allows the dihedral angles between 4-H and 4a-H to be approximately 180°, thus accounting for the large $^3J_{4,4a}$ value of 12 Hz.

From the similar $^3J_{4,4a}$ values of oxytetracycline and its 5,12-diacetate, it is probable that a base conformation such as that of *Figure 5.30* is preferred for both bases.

NMR spectroscopy is of special value in differentiating tetracycline antibiotics, since the functionality array common to each member (see (7))so dominates their appearance and physical properties that electronic and vibrational spectra within the group are similar and of little value for identification purposes [79]. Reviews of the ^{1}H- and ^{13}C-NMR spectroscopy of tetracycline antibiotics are available [75, 80]; these include characteristic features of

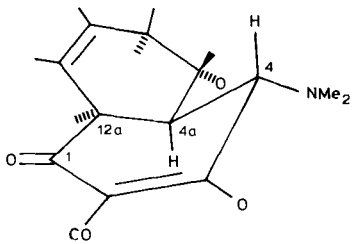


Figure 5.30. Conformational drawing rings A and B of oxytetracycline base. When ring A adopts the half-boat arrangement shown, the dihedral angle between 4-H and 4a-H is approximately 180°.

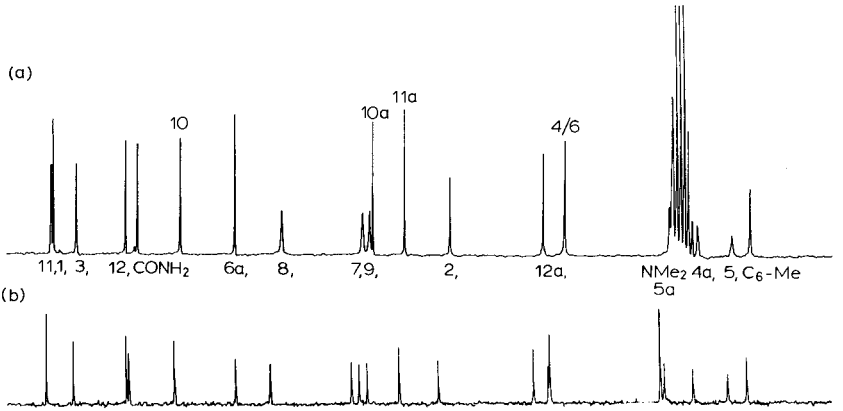


Figure 5.31. Proton noise-decoupled ^{13}C NMR spectrum of tetracycline hydrochloride in (a) DMSO-d_6 and (b) H_2O. The assignments given apply to both spectra. The broad nature and relatively low intensities of resonances C_8, C_7, C_9, C_{4a} and C_5 (due to protonated carbons) in spectrum (a) should be noted, as should the fact that disparities in signal intensities of resonances C_{6a} through C_2 are much smaller in spectrum (b). The NMe_2 and C_{5a} resonances, obscured by the solvent multiplet in spectrum (a), are clearly resolved in spectrum (b).

common products of degradation and isomerization. Thus, the presence of the 4-epimer of tetracycline in a sample of tetracycline hydrochloride may be detected by the appearance of a resonance at 4.75 ppm to low field of the 4-H signal (4.3 ppm) of the antibiotic. Assignment of signals in ^{13}C spectra, for

Table 5.4. T_1 RELAXATION TIMES OF SOME ^{13}C RESONANCES OF TETRACYCLINE HYDROCHLORIDE [80]

Chemical shift (ppm)	T_1 *(s)*[a]	*Assignment*	*H environment*[b]		
			α	β	γ
67.9	1.06 (2.22)	C-6	–	5	3
73.1	1.54 (2.39)	C-12a	–	2	4
95.7	2.83 (3.77)	C-2	–	–	4
106.8	2.09 (2.97)	C-11a	–	1	3
114.4	3.90 (4.58)	C-10a	–	–	3
147.9	2.17 (3.20)	C-6a	–	1	6

[a] In DMSO-d_6 (water value in parentheses).

[b] 

example, that of tetracycline hydrochloride (*Figure 5.31*), was aided by analysis of spin-lattice relaxation times (T_1) measured by the inversion recovery method. If it is assumed that carbons relax from excited spin states predominantly by the dipole–dipole (DD) mechanism (and evidence for this was obtained by measuring NOE factors), then individual T_{1DD} values for quaternary carbons can be related to the counts of β- and γ-protons, thereby aiding spectral assignments [81]. Some results are shown in *Table 5.4*.

BETA-LACTAM ANTIBIOTICS

Proton- and ^{13}C-NMR spectroscopy are likewise powerful tools for the identification of the numerous penicillins and cephalosporins in present-day clinical use, and surveys of such spectral data have been published [82, 83]. Most proton spectra are well resolved at 60 and 100 MHz, but access to higher-field spectrometers is valuable in the case of derivatives of special complexity such as bacampicillin, which is used as a diastereoisomeric mixture (*Figure 5.32*).

Assignments of the ^{13}C-NMR spectra of β-lactam antibiotics provide pertinent examples of the use of fully coupled spectra. Of the three penam ring CH resonances of a penicillin, that at lowest field is assigned to C-3 (attached to the deshielding carboxylate function). There is initial doubt on the C-5 and C-6 assignment, but the C-5 resonance (66.0–68.7 ppm in D_2O) can be differentiated from that of C-6 (57.4–59.1 ppm or 60.0–64.6 ppm for examples with protonated side-chains) by its larger ^1J(CH) value (C-5 near 180 Hz, C-6 near 155 Hz), a difference attributed to the influence of its electronegative neighbours, sulphur (1) and nitrogen (4) [84]. The ^1J(CH) couplings are obtained from a fully coupled spectrum, for example, that of phenoxymethylpenicillin (*Figure 5.33*). Methine and methylene resonances due to side-chain features which occur in the same spectral region (50–75 ppm) as penam ring carbon resonances can be distinguished by their ^1J(CH) values and other means, for example, $ArCHCO_2^-$ of carbenicillin at 61.8 ppm – signal absent after storage in D_2O owing to exchange of CH protons for deuterium. Coupled spectra also aid assignments to the low-field regions of β-lactam spectra, in which several closely placed Cq resonances are found and line splittings are due to long-range interactions.

Thus, in the spectrum of cephazolin (*Figure 5.34*), the quartet at lowest field must arise from the aromatic carbon (C-5) linked to the methyl group of the C-3 substituent and the triplet is due to the lactone carbonyl (equally coupled to 6-H and 7-H); both signals flank the multiplet due to the overlapping resonances of the 4-carboxylate (singlet) and 7-amido carbonyls, and the aromatic C-4 of the 3-CH_2SAr substituent.

Assignment of the C-3 and C-4 carbons of the sulphur-containing ring of cephalosporins is important because of proposals linking their chemical shift difference ($\Delta\delta 4-3$) to antibacterial potency [85], and this has been carried out on the basis of T_1 measurements (C-3 nuclei usually have at least four β-protons and should relax faster than C-4 with no such protons). When the 4-carboxylate function is ionized, C-4 has the lower field resonance but C-3 takes this position when the pH is lowered and 4-CO_2H is unionized, for example, T_1 data for cephalexin in D_2O-TFA: 3.8 s for 137.9 ppm resonance (C-3) and 7.0 s for that at 122.1 ppm (C-4) [83]. It is notable that cephalospor-

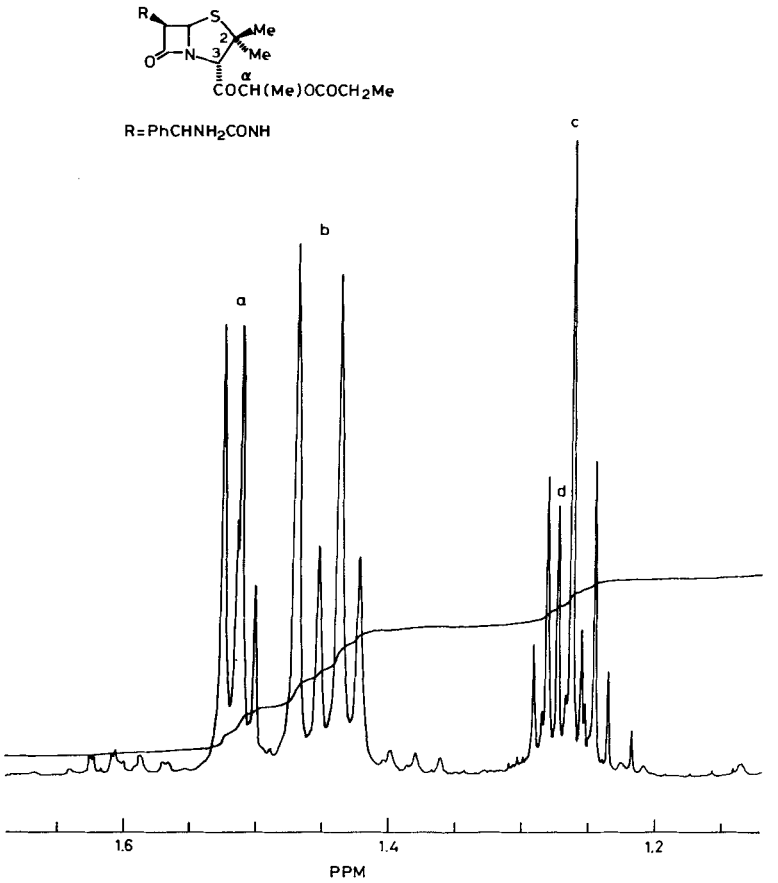


Figure 5.32. 1.0 to 1.6 ppm region of the 400 MHz ^{1}H-NMR spectrum of bacampicillin HCl in CD_3OD. (a) Major and minor α-Me doublet of 3-substituent; (b) major and minor 2α Me singlets (4 lines). Centre of major (c) and minor (d) CH_2Me triplets of 3-substituent.

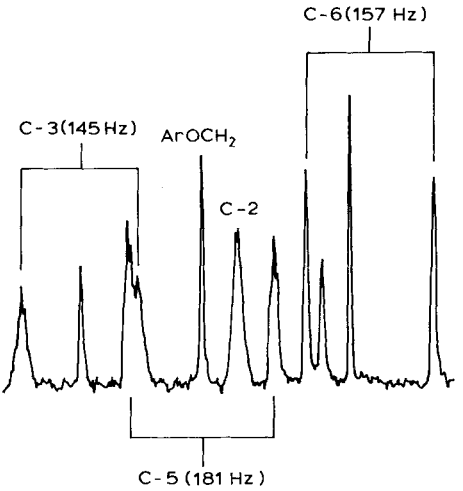


Figure 5.33. The 75–50 ppm region of the proton-coupled ^{13}C-NMR spectrum of the potassium salt of phenoxymethylpenicillin in D_2O.

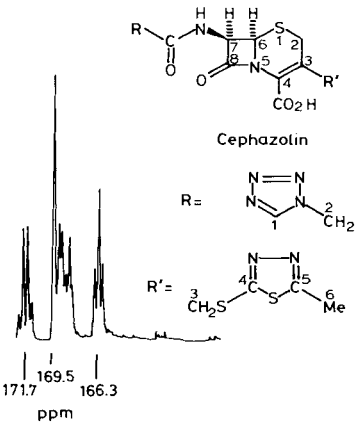


Figure 5.34. The 160–175 ppm region of the proton-coupled ^{13}C-NMR spectrum of the sodium salt of cephazolin in D_2O. The quartet due to the aromatic carbon linked to the methyl group of the C-3 substituent, and the triplet due to the lactam (C-8) carbonyl, both flank the multiplet due to the overlapping resonances of the 4-carboxylate (singlet) and amido carbonyls, and the aromatic C-4 of the 3-CH_2SAr substituent.

ins that have achieved particular clinical success have large $\Delta\delta 4-3$ values (13–16 ppm).

Degradation reactions of β-lactam antibiotics may be monitored by NMR spectroscopy under a variety of conditions, and the data compliment those derived from HPLC investigations and have the virtue of an absolute identification of reaction products, provided NMR data on reference materials are available.

Benzylpenicillin is rapidly converted to (5*R*,6*R*)-benzylpenicilloic acid (8) when dissolved in D_2O containing sodium deuteroxide as seen by absence of the closely-placed 5-H, 6-H doublets (5.4, 5.46 ppm, separation 4 Hz) characteristic of an intact β-lactam ring, while the progressive conversion of the

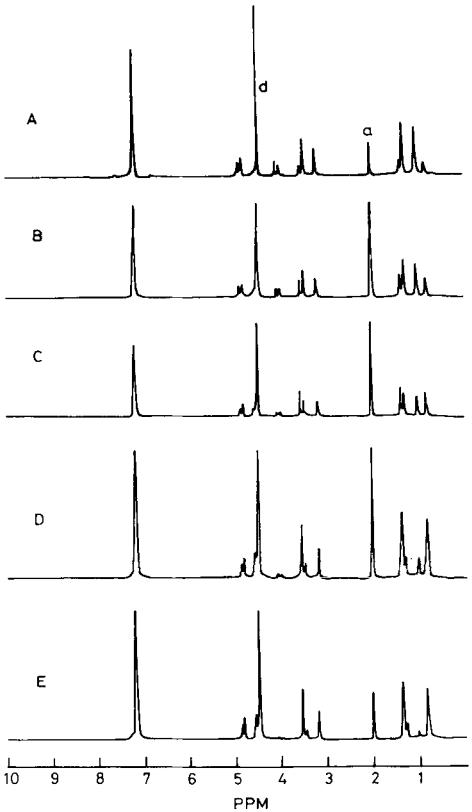
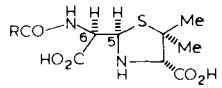
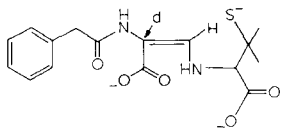


Figure 5.35. ^{1}H-NMR spectra (80 MHz) of benzylpenicilloic acids in D_2O-NaOD showing isomerization of the (5R,6R) diastereoisomer (A) to the (5S,6R) acid (E) at specific time intervals. Key: (A) 6 h; (B) 12 h; (C) 24 h; (D) 48 h; (E) 120 h. Signal (a) is due to acetone and (d) to water (HDO) [89].

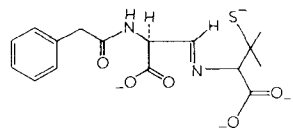


(8)

5*R*,6*R* isomer to (5*S*,6*R*)-benzylpenicilloic acid may be followed by observing changes in the α,β 2-methyl signals in the 1.0–1.6 ppm region (*Figure 5.35*) [86]. Separation of methyl signals of the 5*R*,6*R* isomer is enhanced over that of the parent antibiotic (0.07 → 0.2 ppm) and even more so for the (5*S*,6*R*)-isomer (Δδ 0.52 ppm). At equilibrium, the 5*S*,6*R*-epimer preponderates (*Figure 5.35E*). The fact that deuterium exchange at C-6 does not occur excludes the enamine form of penamaldic acid (9) as an intermediate in the epimerization pathway and supports interconversion via the imine tautomer (10) of the same acid. Magnitudes of the ^3J couplings between 5-H and 6-H of the penicilloic acids confirm *cis* stereochemistry for the 5*R*,6*R* isomer (sep. 5.4 Hz) and *trans* for the 5*S*.6*R* form (sep. 3.0 Hz) [87].



(9) penamaldic acid (enamine)



(10) penamaldic acid (imine)

Spectra of benzylpenilloic acids (see *Figure 5.36*) are more complex than those of the penicilloic acids as a result of the non-equivalence of the methylene protons produced at C-6 after decarboxylation. Full analysis is possible, however, from a 400 MHz spectrum, which reveals four 2-methyl resonances and duplicate 3-H, $PhCH_2$, 5-H and 6-H_2 signals, all evidence of the isomeric nature of the product (*Figure 5.36*). The 6-H_2 signals are both composed of 8 lines as required by the ABX system (CH_2CH), while the 5-H resonances appear as triplets.

Degelaen, Loukas, Feeney, Roberts and Burgen [88] observed changes in the 270 MHz ^{1}H-NMR spectra of benzylpenicillin in DCl-D_2O at pH 2.5 and 37 °C and made assignments to the various degradation products by comparisons with spectra of standards recorded under the same conditions. In the early stages, signals due to benzylpenillic, penicilloic and penamaldic acids appeared almost simultaneously, diagnostic resonances being 5.9 ppm (5-H), 1.26, 1.39 ppm (2-Me) and 7.78 ppm (5-H), respectively. Most of the penicillin had degraded after 100 min as judged by intensities of the 5.45 and 5.53 ppm resonances (5-H, 6-H) and an apparent first-order rate constant of 0.44 m^{-1} was calculated from integral data. After day 1, lines due to penilloic acid

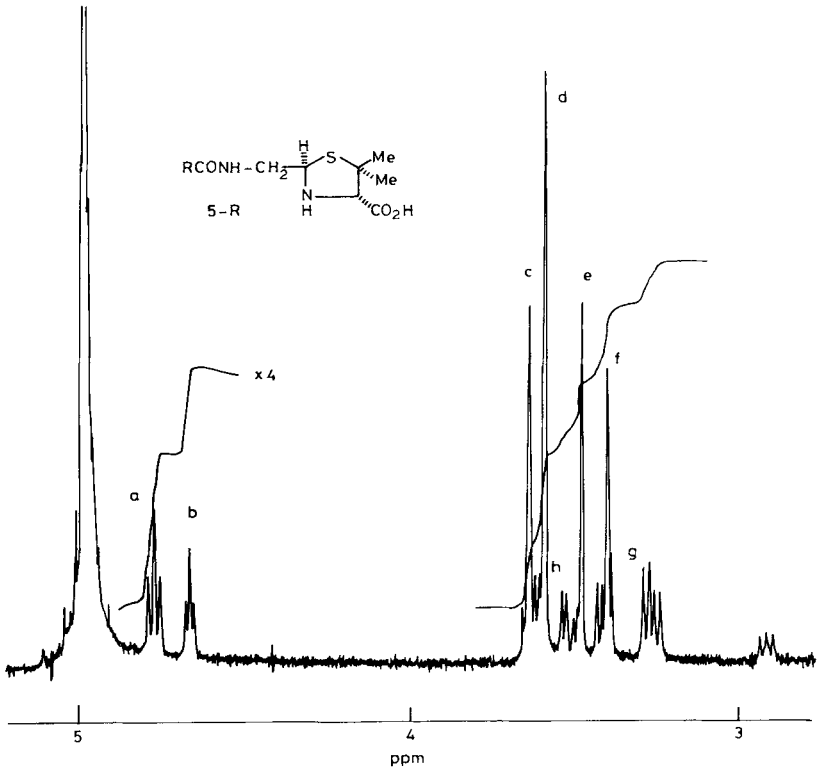


Figure 5.36. 3 to 5 ppm region of the 400 MHz ^1H spectrum of benzylpenilloic acids in D_2O-$NaHCO_3$ recorded 48 h after preparation of the solution: a, b *major and minor 5-H triplets;* c, d *minor and major $PhCH_2$ singlets;* e, f *major and minor 3-H singlets;* g *approximate centre of major 6-CH_2 signal (8 lines, one obscured by* f*);* h *approximate centre of minor 6-CH_2 signal (several lines obscured).*

appeared which steadily increased with concomittant fall in the intensities of signals due to the initially formed acids. After 30 days the spectrum was essentially that of penilloic acid. A spectrum recorded at Bath at 400 MHz is provided as an illustration (*Figure 5.37*); at this frequency the 2-methyl signals are resolved sufficiently to permit quantitative analysis (see *Figure 5.37B*). If the short-lived benzylpenicillenic acid is a common precursor, as proposed by others [89], then all products derived from it should carry a deuterium atom at C-6. However, examination of 5-H and 6-H resonances of penillic and penicilloic acids formed in the degradation showed only 30–35% deuterated material to be present, evidence that these acids must also result from other pathways. Thus the 5-H signal of benzylpenillic acid (11) (signal b of

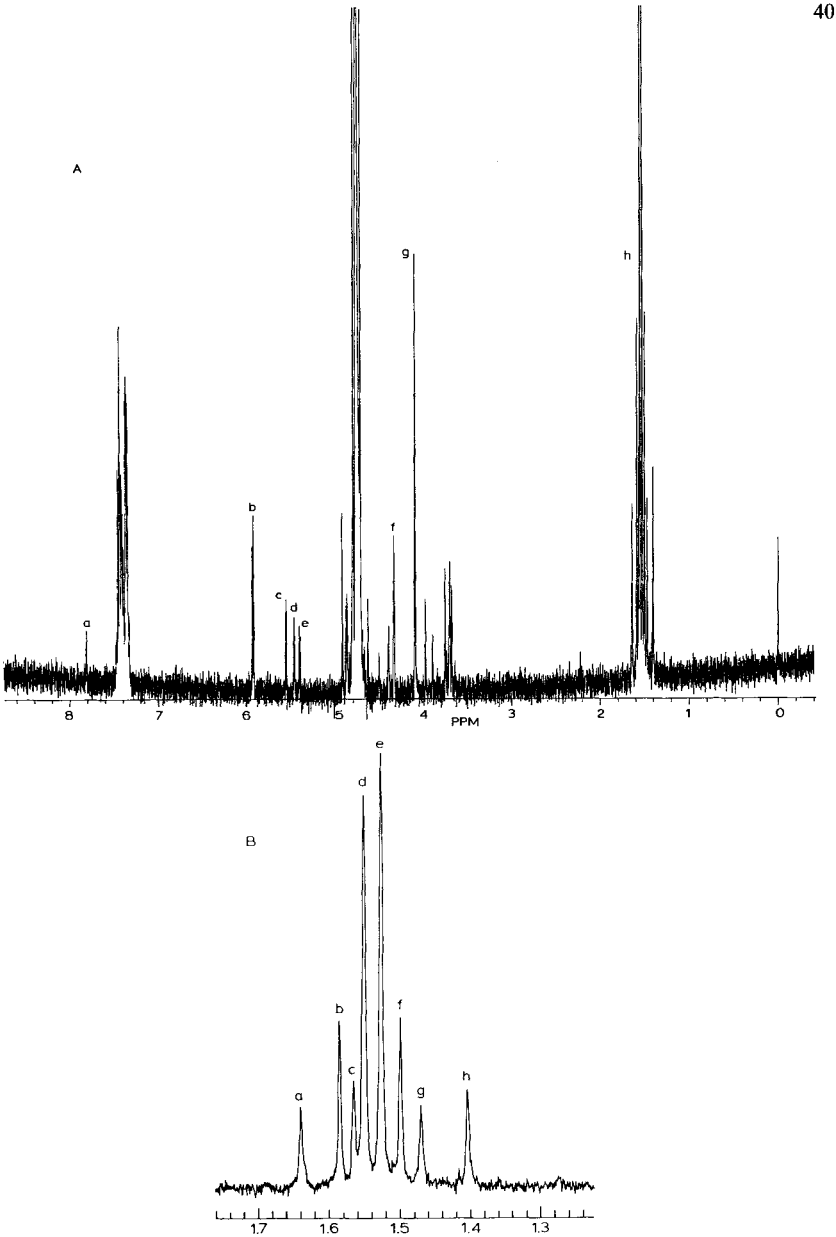
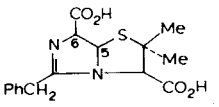


Figure 5.37. 400 MHz ^{1}H-NMR spectrum of benzylpenicillin sodium (5 × 10^{-3} M) in D_2O-DCl at pH 2.52, 25 °C recorded 75 min after preparation. (A) Complete spectrum. (a) Penamaldic acid (5-H); (b) penillic acid (5-H); (c,d) penicillin (5-H, 6-H); (e) (5R,6R)- and (5S,6R)-penicilloic acids (5-H); (f) penicillin (3-H); (g) penillic acid (3-H); (h) 2-Me signals (see B). (B) Expansion of 2-Me region. (ag) penamaldic acid; (b, f) penicillin; (c,h) (5R,6R)-penicilloic acid; (d,e) penillic acid.



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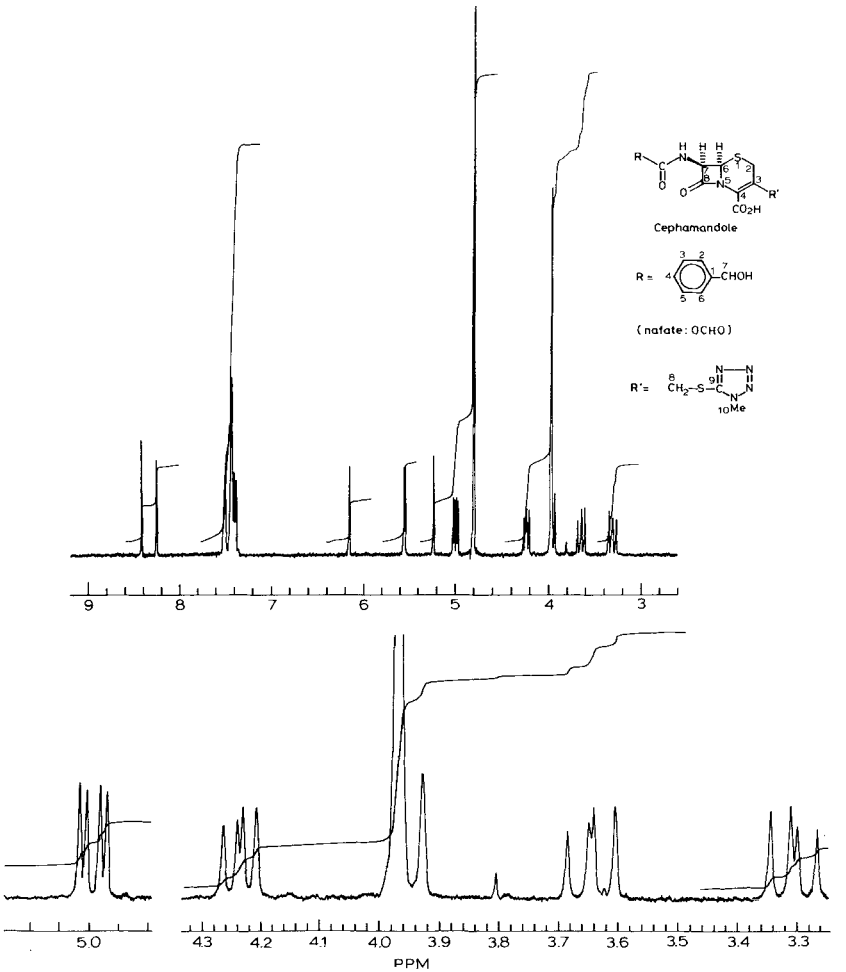


Figure 5.38. 400 MHz ^1H spectrum of a freshly prepared solution of cephamandole nafate (Kefadol) in D_2O. Signal duplication is evident throughout the spectrum due to rapid hydrolysis of the nafate ester to the free alcohol. The two lowest-field signals are due to COCH of formate and the intact ester. The expansion shows duplicate 6-H (or 7-H) (near 5 ppm), CH_2S (one proton of pair, near 4.25 ppm), and 2-CH_2 (near 3.65 and 3.3 ppm) resonances.

Figure 5.37A) is composed of an overlapping doublet and singlet which arise from the 6-H and 6-D derivatives, respectively. ^{1}H-NMR has also aided study of the breakdown of mecillinan [90] and the technique clearly has high potential for studies of this kind.

Examples of related work involving cephalosporin antibiotics are the conversion of cephamandole nafate (Kefadol) to cephamandole (*Figure 5.38*) – remarkably distinct chemical shift differences are found for corresponding protons of the ester and free acid at positions remote from the benzylic centre,

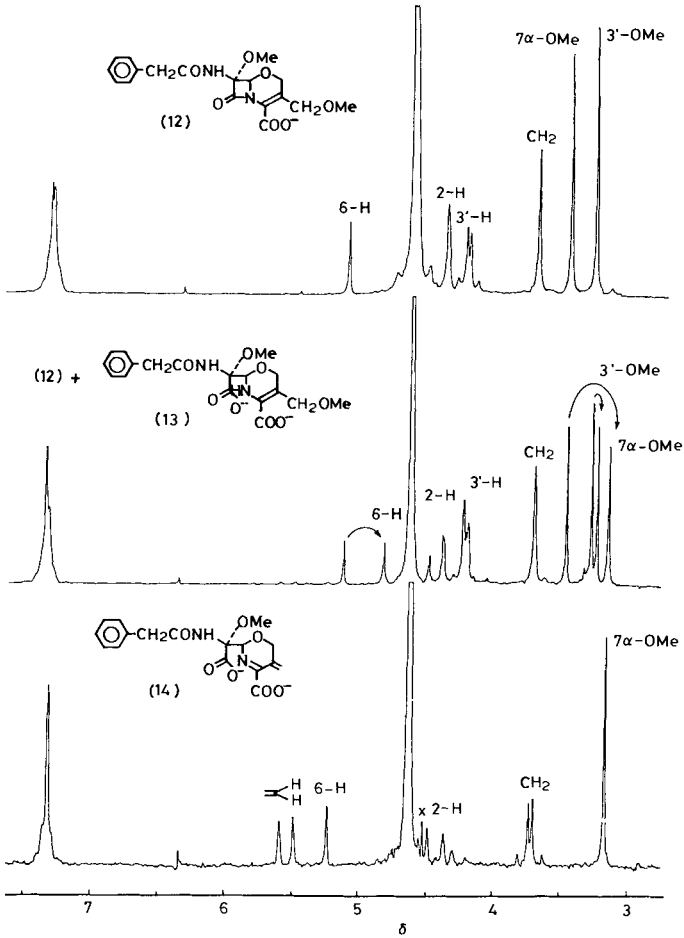


Figure 5.39. 200 MHz ^{1}H-NMR spectra of the oxacephem (12) and its degradation products (13) and (14) in D_2O.

and studies of the breakdown of oxacephem derivatives such as moxalactam [91].

The derivative (12) (*Figure 5.39*) in D_2O at p*D* 10.4 and 35° first degrades to the ring-opened product (13) (detected by upfield shifts of the 6-H and 7-OMe signals), and then to the *exo*-methylene compound (14) formed by loss of the leaving group attached to C-3 methylene and detected by appearance of a vinylic signal near 5.5 ppm. The rate constant for the ring opening of moxalactam was readily established by following changes in the relative intensities of the 7- OMe resonances (*Figure 5.40*). Similar work has been reported by Pratt and Faraci [92].

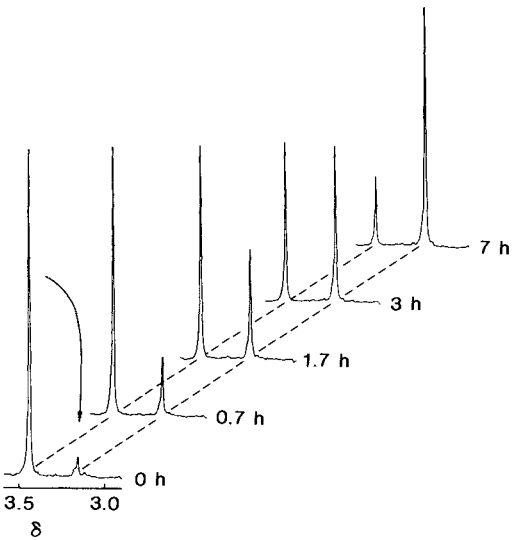


*Figure 5.40. Changes in the intensities of ^1H of methoxy NMR signals of moxalactam (3.47 ppm) and its ring-opened product (3.17 ppm) during degradation of the antibiotic in deuterated carbonate buffer at p*D *10.4 and 35 °C.*

ERYTHROMYCIN

NMR analysis of spectra of erythromycin (15) provides an example more complex than those of the antibiotics so far discussed (there are 41 ^1H resonances to be resolved and assigned), and this has now been achieved using 2D chemical shift correlation methods [93]. An expansion of part of the 400 MHz COSY-45 ^1H plot is shown in *Figure 5.41*, together with the analysis

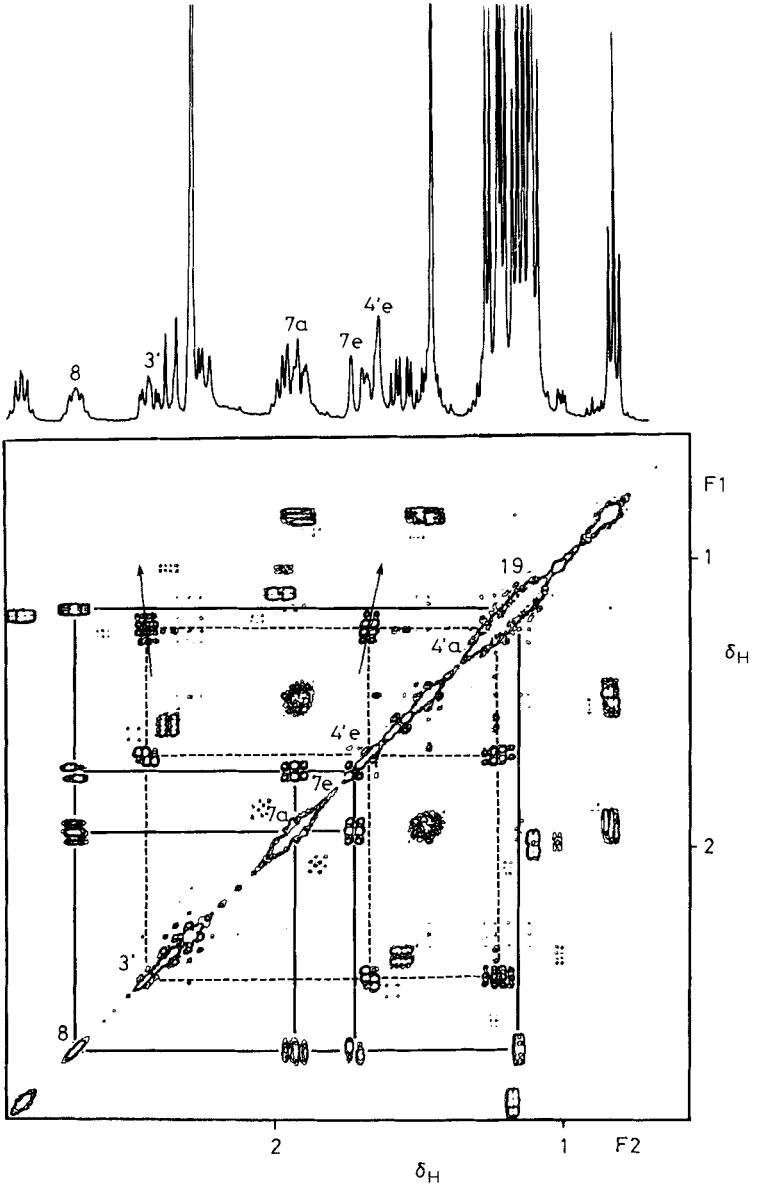
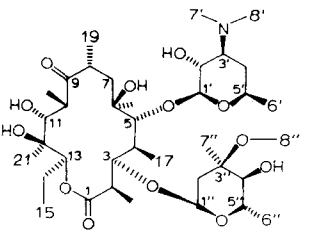


Figure 5.41. An expansion of the high-field quadrant of the 2D ^1H COSY-45 NMR spectrum of erythromycin in $CDCl_3$ as a contour plot beneath the corresponding 1D spectrum. The solid lines and broken lines trace out the cross-peaks due to J connectivities in the 19-CH_3, 8-H, 7ax-H, 7eq-H and the 4'ax-H, 4'eq-H, 3'-H spin systems, respectively. The arrows on two of the cross-peaks serve to indicate their opposite slopes.



(15)

of connectivities in the 19-H, 8-H, 7eq-H, 7ax-H spin system (bold lines). This isolated CH_3–CH-CH_2 spin system is unique in the molecule and could be assigned unambiguously on this account. In certain spin systems, *slopes* of the cross-peaks gave the relative signs of coupling constants and hence allowed differentiation of geminal (^2J) and vicinal (^3J) interactions. Thus in the 3′-H, 4′eq-H, 4′ax-H system (broken lines of *Figure 5.41*), the 4′ax resonance (1.22 ppm) gives a cross-peak slope with 4′eq (^2J) that is opposite in sign to the slope of its interaction with 3′-H (^3J). Another plot was a 2D ^{13}C,^1H spectrum (*Figure 5.42*) which allowed directly bonded ^1H and ^{13}C nuclei to

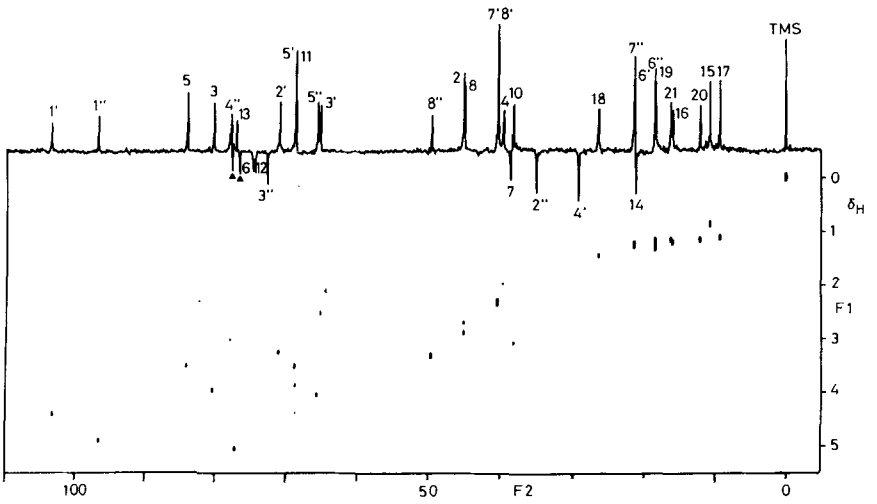


Figure 5.42. The 2D ^{13}C,^1H COSY NMR spectrum of erythromycin in $CDCl_3$ as a contour plot beneath the corresponding 1D ^{12}C spin-echo NMR spectrum. The spin-echo spectrum (pulse sequence: $90°-(\tau-180°-\tau)_n$-data acquisition) was acquired with broadband proton decoupling during the second period and data accumulation. With τ = 8 ms CH_2 and C resonances are inverted relative to CH_3 and CH resonances. The small triangles (△) indicate the two outer lines of the solvent triplet. The contour plot levels are higher than the cross-peaks due to methylene moieties.

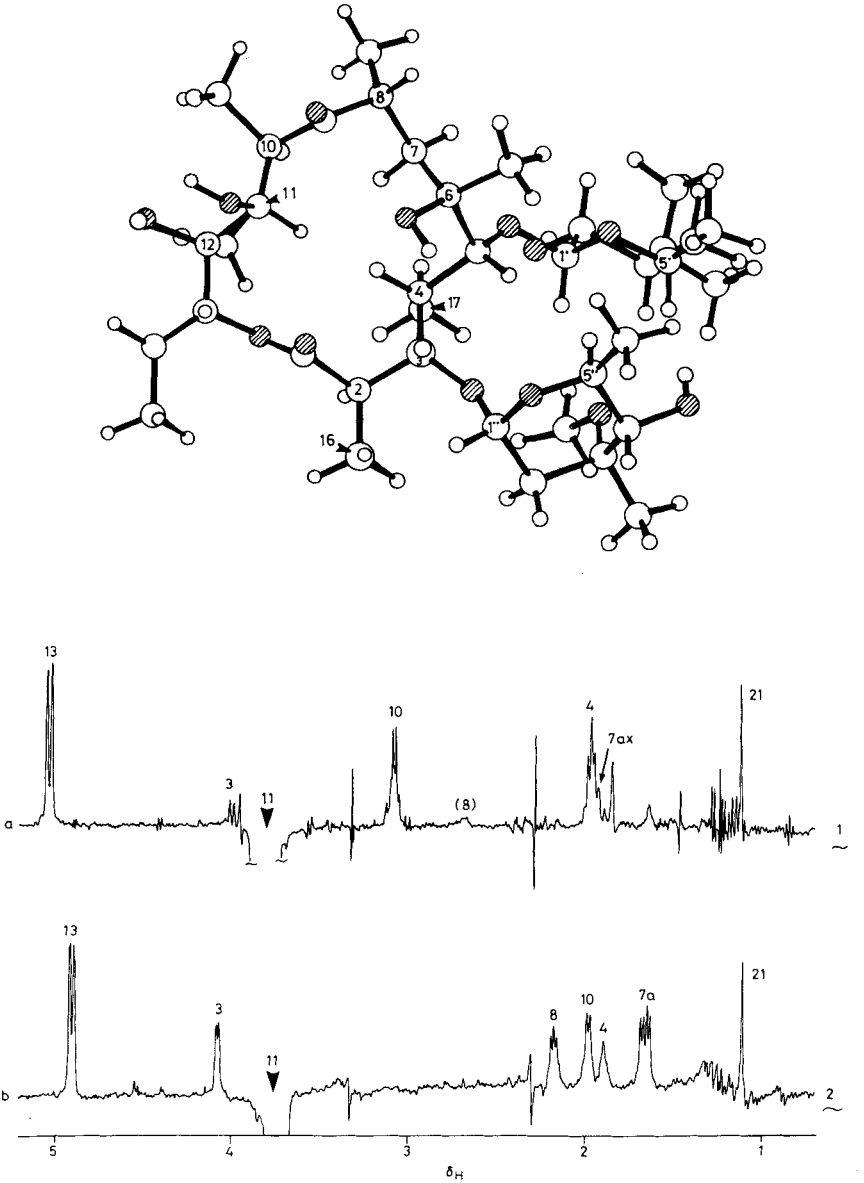


Figure 5.43. 400 MHz NOE difference spectra obtained by irradiation of H-11 in erythromycin (1) and its analogue with C-9 carbonyl reduced to CH_2 (2). Solid state conformation of erythromycin is shown above.

be identified. In this presentation, CH_2 and Cq resonances are inverted relative to CH_3 and CH signals. With ^1H proton assignments already made, those of ^{13}C could be read off directly. A relayed coherence transfer experiment (^{13}C,^1H) [94] resolved ambiguities (due to ^1H spectral overlap) remaining from the 2D ^1H,^{13}C COSY experiment.

Extensive NOE difference experiments provided evidence that the solution conformation of erythromycin in deuterochloroform is close to that of the crystalline hydroiodide dihydrate (NOEs should be observed between protons 3 Å apart) [95]. The NOED spectrum due to irradiation of 11-H is shown in *Figure 5.43*. NOEs to 4-H, 7ax-H, 10-H, 13-H and 21-Me attached to C-12 were clearly observed and were all compatible with the solid-state conformation included as part of *Figure 5.43*. A small NOE between 11-H and 3-H was unexpected because of the distance between the two protons in the solid-state (about 3.7 Å), and it was concluded that it resulted from a m inor folded in lactone ring conformation in fast equilibrium with a major folded out conformer. The analogue of erythromycin with the C-9 carbonyl reduced to methylene gave an NOE between protons 8 and 11 unobserved in the parent, while a clear 3,11 interaction was also apparent. It was proposed that the dihydro analogue existed in solution as a mixture of lactone ring conformations of the folded in and folded out variety in fast exchange such that 3-H and 11-H were spatially proximate.

Gentamicin is a mixture of aminoglycosides composed of three major (C_1, C2, C_{1a}) and several minor components (*Figure 5.44*). Proton-NMR has been used to control the ratios of the chief components within broad limits, but is unsuitable as a specific assay because of extensive overlap of resonances [96]. However, since a ^{13}C-NMR spectrum includes well resolved signals specific for each of the major gentamicins (*Figure 5.45*), the technique may be employed to provide ratios of the major components and as a means of quantitative analysis [97, 98]. In contrast to ^1H-NMR, the intensities of ^{13}C resonances usually correlate poorly with spin populations. The reasons are (1) variation in the relaxation times of the carbon atoms of the molecule, (2) differential NOE factors, and (3) instrumental effects [99]. Allowance was made for (1) by introducing a delay between pulses long enough to permit complete relaxation of the spin system ($> 5 \times$ longest spin-lattice relaxation time of the analytical carbons). Differential NOE effects were avoided by adopting a procedure based on the relationship between peak height ratios of the analyte to standard (dioxan) resonance intensities, and analyte concentration. Pure samples of C_1, C_{1a}, and C_2 gentamicin bases were obtained by an HPLC procedure and used to construct calibration plots and derive response factors. Analyses were based on fully relaxed spectra, and on spectra recorded under

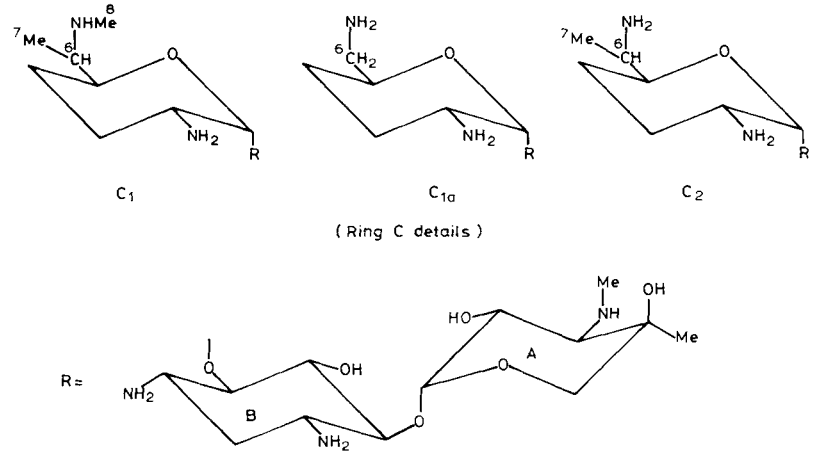


Figure 5.44. The structures of gentamicins C_1, C_{1a} and C_2. The C_{2a} member is the C-6 epimer of C_2.

steady-state conditions, which were less time-demanding although probably less accurate. The order of accuracy achieved was low compared with the HPLC method. However, improved results have been reported by Busson, Claes and Vanderhaege [98], who used the sulphate salts in a method based on peak height measurements of selected resonance signals obtained under steady-state conditions and using relative response factors for correction of different T_1 values and nuclear Overhauser enhancements. The ratios of four components (as above plus C_{2a}) were obtained from this work. The crucial peak height measurements were obtained from the computer listings.

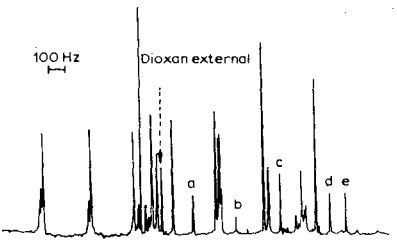
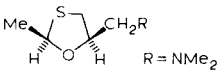


Figure 5.45. 22.5 MHz ^{13}C-NMR spectrum of the total base (150 mg) recovered from a commercial sample of gentamycin sulphate in D_2O (1 ml) with external dioxane as standard. Analytical resonances expressed in ppm from TMS employing 67.4 ppm as the chemical shift of dioxane are: C_1 (a) C-6 near 58, (c) C-8 near 34, and (e) C-7 near 15; (b) near 46; C_2 (d) near 19 ppm.

CHIRAL RESOLUTION

The use of NMR for establishing the composition of antipodal mixtures has been much aided by the introduction of chiral shift reagents [100] and their use is practicable provided that chemical shift differences between related resonances are great enough at 60 or 100 MHz to allow base-line resolution. A recent example is the analysis of *R,S* mixtures of the thio analogue of muscarine base (16) [101].

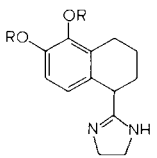


(16)

In the presence of a molar proportion of $Eu(tfc)_3$, antipodal 2-methyl resonances were resolved at 60 MHz and enabled a correlation to be made between the *R*:*S* ratio and optical rotation which (by extrapolation) provided the specific rotational [α] value indicative of complete resolution to a reasonable approximation.

Unfortunately, the line-broadening effects of chiral shift reagents based on lanthanide elements (due to paramagnetic relaxation) are directly proportional to the strength of the operating field and hence resolution is generally lost in spectra recorded above 100 MHz. Cobalt-derived shift agents do not, however, suffer from this disadvantage and the cobaltous ATP chelate is of special value, since is may be applied to aqueous (D_2O) solutions [102]. The compound has recently been used to assess the optical purity of enantiomers of the imidazoline derivatives (17a) and (17b) [103]. The 360 MHz spectrum of racemic hydrochlorides in D_2O (*Figure 5.46*) showed duplicate aromatic signals which were sufficiently resolved to allow estimates of the enantiomeric excess (> 98%) of resolved materials.

Another approach to antipodal analysis is that of derivatization with chiral reagents, use of which is subject to the proviso that they must be known to



(17) a, R = H
b, R = Me

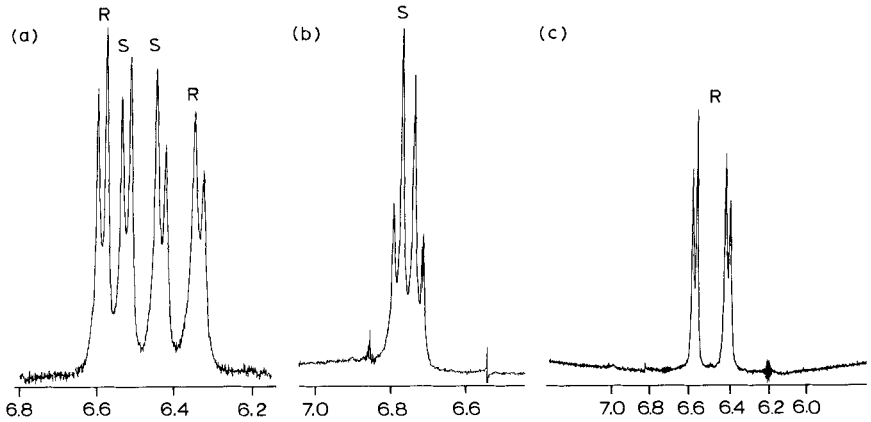
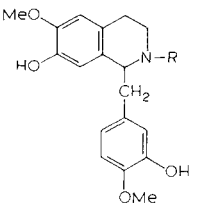


Figure 5.46. Part of the 360 MHz ^{1}H-NMR spectra of (a) racemic (17a) in D_2O + 200 µl CoATP, (b) (−)-(S)-antipode in D_2O + 300 µl CoATP, and (c) (+)-(R)-antipode in D_2O + 150 µl CoATP. Resonances are due to the aromatic protons of (17a).

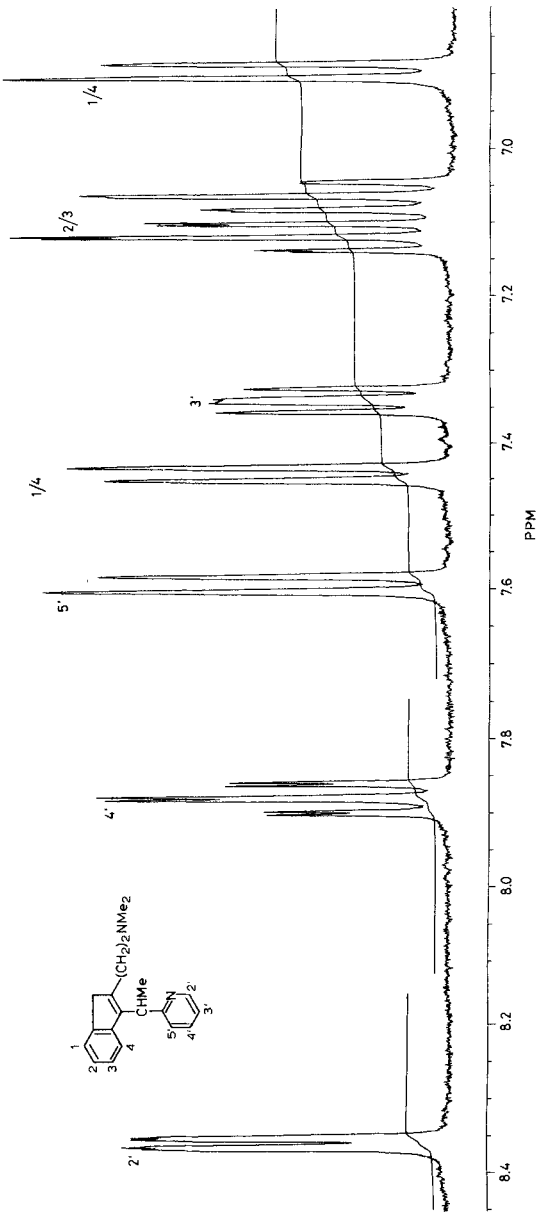
be optically pure themselves. Chemical shift differences between the resultant diastereoisomers often occur, and such differences are enhanced by recording spectra under high-field conditions. Thus Rice and Brossi [104] measured the optical purity of the tetrahydrobenzylisoquinoline (18) by reacting mixtures with *S*-(−)-α-methylbenzyl isocyanate and examining the ^{1}H-NMR spectra of the diastereoisomeric ureas (19); the isomeric methyl signals (Me) showed a separation of 0.25 ppm (55 Hz) at 220 MHz. The authors claimed that less than 1% of a minor antipode could be detected in a mixture by this means.



(18) R = H
(19) R = CONHCH(Me)Ph

USE OF CYCLODEXTRINS

The existence of inclusion complexes formed between annular cyclodextrin molecules with 6 (α), 7 (β) or 8 (γ) dextrose (glucose) units and a variety of guest molecules is well known [105]. When included molecules are chiral,



(upper spectrum)

(lower spectrum)

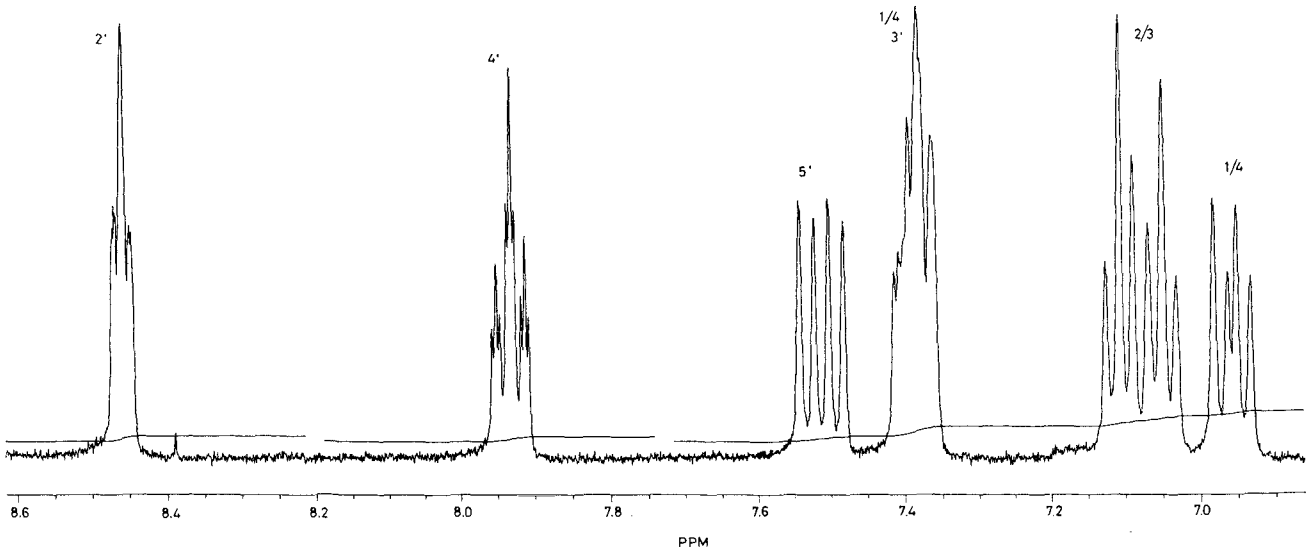


Figure 5.47. Low-field region of the 400 MHz ^{1}H-NMR spectrum of (R,S)-dimethindene maleate in D_2O. Upper spectrum, no additive; lower spectrum, after addition of 1 molar proportion (approx) of β-cyclodextrin. Clear duplications of 5' and 1 (or 4) proton signals are evident, while most of the other aromatic resonances become more complex after addition of β-cyclodextrin.

diastereoisomeric complexes result which may exhibit different physical properties. An early example was provided in 1959 by observation of the partial resolution of mandelic acid by crystallizing the racemic acid in the presence of an excess of β-cyclodextrin [106]. The fact that such differences extend to ^{1}H-NMR chemical shifts (as we have established by recording the spectrum of (*R,S*)-mandelic acid in the presence of a molar proportion of β-cyclodextrin: the benzylic proton signal formed a narrow doublet, separation 1 Hz at 270 MHz in D_2O) provides a simple and rapid means of establishing the extent of diastereoisomeric relationships between cyclodextrins and an antipodal pair of guest molecules, and has potential value as a means of determining optical purity. There have been several reports of NMR differences between the antipodal components of racemic mixtures seen in the presence of a cyclodextrin [107, 108] and we have observed the phenomenon for a variety of chiral medicinal agents, including dimethindene and carbinoxamine (antihistaminics) and the central analgesics methadone and alphaprodine (see below).

Antipodal analysis by NMR employing cyclodextrins has several distinct advantages over derivatization and chiral shift reagent methods, namely:

(1) the material is water- (and D_2O-) soluble, so can be applied directly to water-soluble chiral agents, as represented by many pharmacologically active therapeutic agents (for example, protonated basic salts or alkali metal salts of acids);
(2) cyclodextrins have no broadening effects on ^{1}H resonances, and so, full advantage may be taken of the superior resolving power of very high frequeny (270 MHz and above) spectrometers (cf. use of lanthanide shift reagents whose paramagmetic relaxation effects are directly proportional to the operating frequency);
(3) the ^{1}H chemical shift range of cyclodextrins is narrow (about 3.5 to 5.1 ppm) and, in particular, does not overlap the aromatic and alkenic or higher-field alkyl proton regions.

The ^{1}H-NMR spectrum of (*R,S*)-dimethindene maleate before and after addition of a molar proportion of β-cyclodextrin is a good example of how antipodal resonances may be resolved by this means (*Figure 5.47*). While effects are seen chiefly amongst the aromatic protons, the methine proton signal adjacent to the pyridyl substituent is also duplicated in the presence of β-cyclodextrin. Spectral changes induced by α- and γ-cyclodextrin were much less pronounced; hence, the 7-ring molecule (β) must possess the optimal dimensions for accommodation of the guest molecule in this case.

β-Cyclodextrin-induced changes amongst the aromatic resonances of carbinoxamine tartrate were also observed, but those involving the benzylic

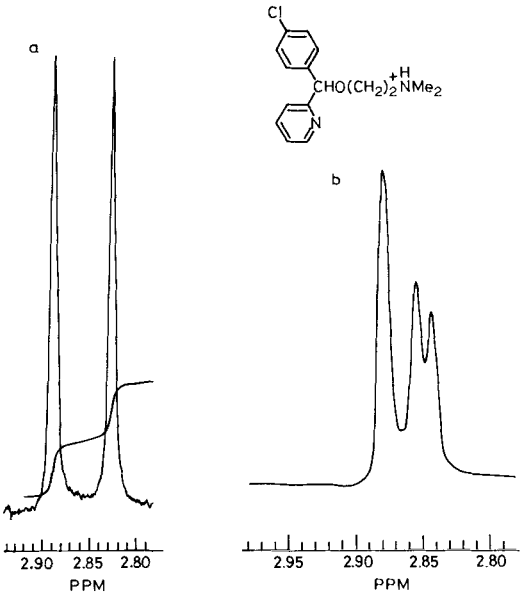


Figure 5.48. Part of the 400 MHz ^{1}H-NMR spectra of (R,S)-carbinoxamine tartrate in D_2O showing the non-equivalent N-methyl signals (a) in D_2O alone; (b) in D_2O plus 1 mole equivalent of β-cyclodextrin – note duplication of the higher-field resonance.

methine resonance and dimethylamino signals were of special interest (*Figure 5.48*). The benzylic signal (not shown) was split into a doublet-line and separation was greatest when γ-cyclodextrin was employed as the host molecule ($\Delta\delta \approx 12$ Hz at 400 MHz); hence, this signal offers the best means of monitoring optical purity, since base-line resolution is possible. The H^+NMe_2 signal of the free carbinoxamine salt formed a pair of well-separated lines (thus the two *N*-methyl groups must be magnetically non-equivalent in the preferred solute conformation of the molecule), the higher field member of which split into a doublet after addition of β-cyclodextrin.

Two chiral opioid analgesics have been examined. Inclusion of (*R,S*)-α-aprodine into β-cyclodextrin resulted in increased complexity of its aromatic proton resonances (unresolved) and clear duplication of the ester methyl and 3-methyl signals to produce overlapping triplets and doublets, respectively (*Figure 5.49*). In the case of (*R,S*)-methadone hydrochloride, the methyl signal of the propionyl function (which appeared as a singlet in D_2O due to deuterium exchange of the adjacent CH_2 protons) formed a pair of lines (separation

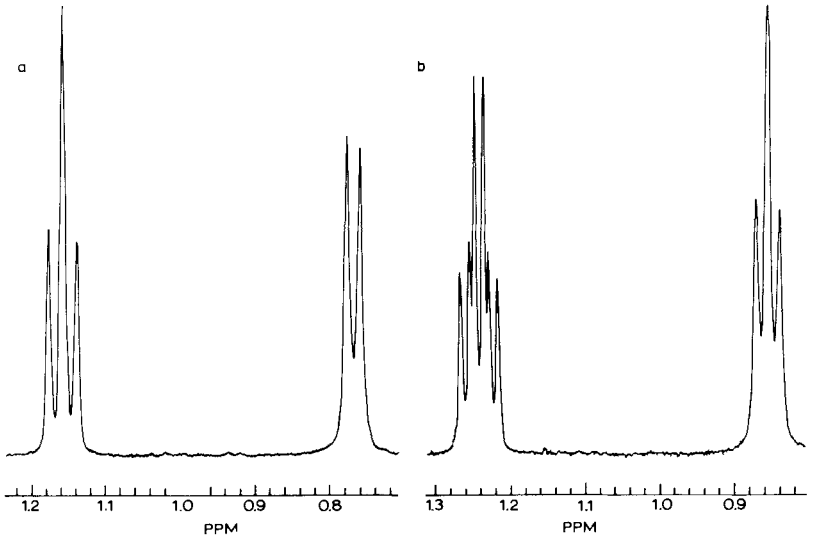


Figure 5.49. Part of the 400 MHz ^{1}H-NMR spectra of (R,S)*-alphaprodine hydrochloride showing the 3-methyl (near 0.8 ppm) and ester methyl (OCH_2 Me, near 1.2 ppm) resonances (a) in D_2O alone; (b) in D_2O plus 1 molar equiv. of β-cyclodextrin (see* Figure 5.3 *for formula).*

6.3 Hz at 400 MHz) after addition of β-cyclodextrin, while one of the β-methylene proton resonances formed an overlapping multiplet (*Figure 5.50*).

DRUG METABOLISM

The relative insensitivity of CW NMR precluded its use for the determination of endogenous and exogenous metabolites in body fluids. In addition, the resolving power of low-field instruments was insufficient to enable the assignment and identification of such complex mixtures. While NMR has been used to elucidate the structure of unknown drug metabolites extracted and isolated from body fluids, the improved sensitivity and resolution of modern high-field spectrometers has allowed the direct determination of compounds in biological materials. The chief advantages of NMR are the possibility of simultaneous quantification and identification of individual compounds, the measurement of several components of a mixture at the same time, and the minimal pretreatment of sample required.

The ordinary proton spectra of body fluids such as urine or serum present various problems which must be overcome before useful spectra can be

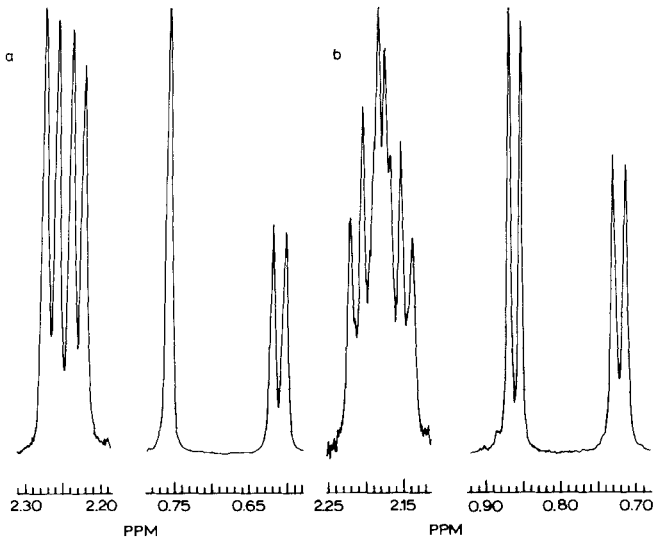


Figure 5.50. Part of the 400 MHz ^{1}H-NMR spectra of (R,S)-methadone hydrochloride ($Me_2NCHMeCH_2CPh_2COCH_2Me \cdot HCl$) showing one of the β-methylene (near 2.2 ppm), methyl of the terminal propionyl group (near 0.8 ppm, a singlet because the adjacent methyl protons are rapidly deuterated) and the α-methyl (near 0.6 ppm) resonances, (a) in D_2O alone; (b) in D_2O plus 1 mole equiv. of β-cyclodextrin.

obtained. The major one is the presence of a large signal from water which restricts the dynamic range of the spectrum and prevents accurate integration of smaller peaks due to metabolites. Several methods have been used to eliminate this signal. One of the simplest procedures is to saturate the water resonance by gated irradiation of the solvent resonance [109], as used to obtain the first informative spectra from serum by Bock [110]. *Figure 5.51* shows the ^{1}H spectrum of a urine sample after 48 scans with homo-gated irradiation (with the decoupler off during data aquisition) of the water resonance at about 5 ppm. This figure shows the large number of endogenous metabolites which can be observed in urine by NMR under these circumstances. Signals occurring near the solvent peak may also be affected by the decoupling.

Other approaches to elimination of the water signal have exploited the difference in relaxation times between water and the compounds of interest, and have the advantage that peaks near the solvent are unaffected. For example, WEFT (Water Eliminated Fourier Transform) NMR uses an inversion recovery pulse sequence (designed for T_1 measurement),

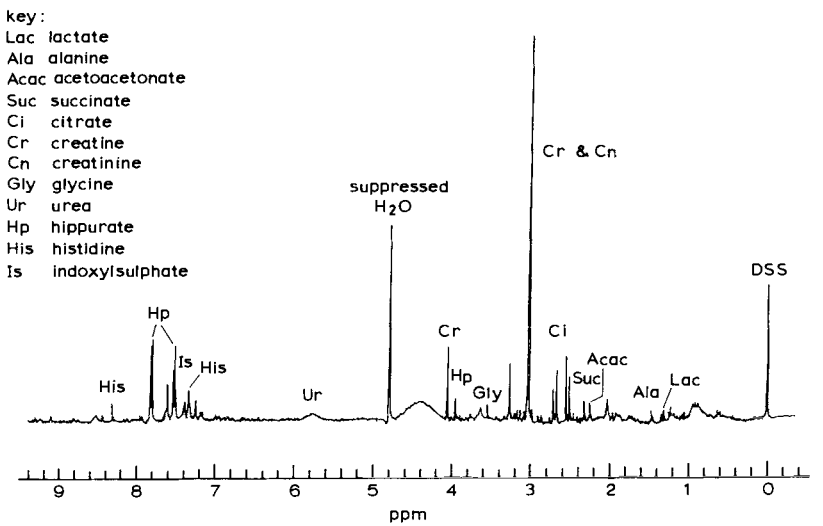


Figure 5.51. 400 MHz ^{1}H-NMR spectrum of human urine with 10% D_2O added as lock signal. The water resonance was suppressed by homo-gated secondary irradiation. 48 scans were accumulated. The 16K data points used to collect the spectrum were zero-filled to 32K and an exponential function corresponding to a 1 Hz line broadening was applied to the FID.

180°–τ–90°–data acquisition [9] in which the time interval, τ, is chosen such that the water resonance, which is expected to have the longest T_1 in the sample, has zero magnetization after the 90° pulse [113, 114]. The CPMG spin-echo pulse sequence 90°–(τ–180°–τ)n–data acquisition (n = number of repetitions), has been used with the pulse interval, τ, adjusted to attenuate the water signal, for example, from erythrocyte and protein suspensions [113]. The technique is improved by the addition of ionic species such as ammonium chloride which increases the chemical exchange of the water protons and thus shortens T_2 relative to the compounds of interest. This method is known as WATR (**W**ater **A**ttenuation by T_2 **R**elaxation) [114]. Solvent suppression can also be achieved by selective excitation of the spectrum with special pulses such that the water resonance occurs at a point of null excitation [115–119]. However, distortion of peaks near the null point may occur.

Another problem which occurs in spectra of serum and plasma samples is broad resonances arising from the relatively immobile high-molecular-weight species such as proteins and lipids which have higher values of T_2. The Hahn spin-echo sequence [120] (which does not include the refocusing pulses used in CPMG to overcome magnetic field inhomogeneity) was used initially in the

study of erythrocyte metabolites [121] and subsequently for the detection of normal and abnormal metabolites in serum, plasma and urine of human and experimental animals [122–125]. A pulse interval of about 60 ms is usually required to remove the broad resonances while retaining the signals of lower-molecular-weight metabolites. In addition, this interval causes doublets with a coupling constant of about 8 Hz to invert; this can aid spectral assignment, but may make quantification less accurate.

Drug metabolites can be analysed when they have resonances which occur in regions of the urine proton spectrum normally clear of signals from endogenous compounds. This has been exploited by groups working on the metabolism of paracetamol [126–128], oxpentifylline (which is used in the treatment of vascular disease [129]) and metronidazole [130]. A novel ampicillin metabolite, a 1,4-diketopiperazine previously observed only after *in vitro* incubation with serum albumin, has been detected directly in rat urine [131]. Two-dimensional spectroscopy may aid the identification of metabolites by increasing the spectral resolution and Bales, Nicholson and Sadler have used ^1H–^1H correlation spectroscopy to examine urine after ingestion of paracetamol [132].

Figure 5.52 shows the proton spectrum, with secondary irradiation of the water resonance, of urine from a patient who had taken a paracetamol overdose. It shows the main metabolites, the glucuronide and sulphate, and also ethanol taken at the same time as the tablets. For quantitative measurements, longer than usual pulse delays (about 5 s) are necessary to ensure complete relaxation of resonances between pulses. Sufficient data points must be used to give good digital resolution but zero-filling can be used so that the time required to accumulate the spectrum is halved while obtaining the same resolution. Resolution enhancement can be achieved by multiplying the FID before transformation by a function which de-emphasises its beginning, for example, a negative exponential or a Gaussian function. A choice of the most commonly applied window functions is usually included in the software packages of commercial NMR spectrometers.

Drugs need to be present in the millimolar concentration range for detection. In cases where the sensitivity of the FT NMR method is still insufficient, it may be possible to pre-concentrate the sample in some way. Freeze-drying the sample and redissolving in deuterated water and a simple chromatographic procedure using Bond-Elut columns (**S**olid **P**hase **E**xtraction **C**hromatography, SPEC-NMR) have been used for naproxen [133] and ibuprofen measurements [134].

Studies have not been restricted to the ^1H nucleus. Other nuclei have advantages when they are not as common as hydrogen or when they are not

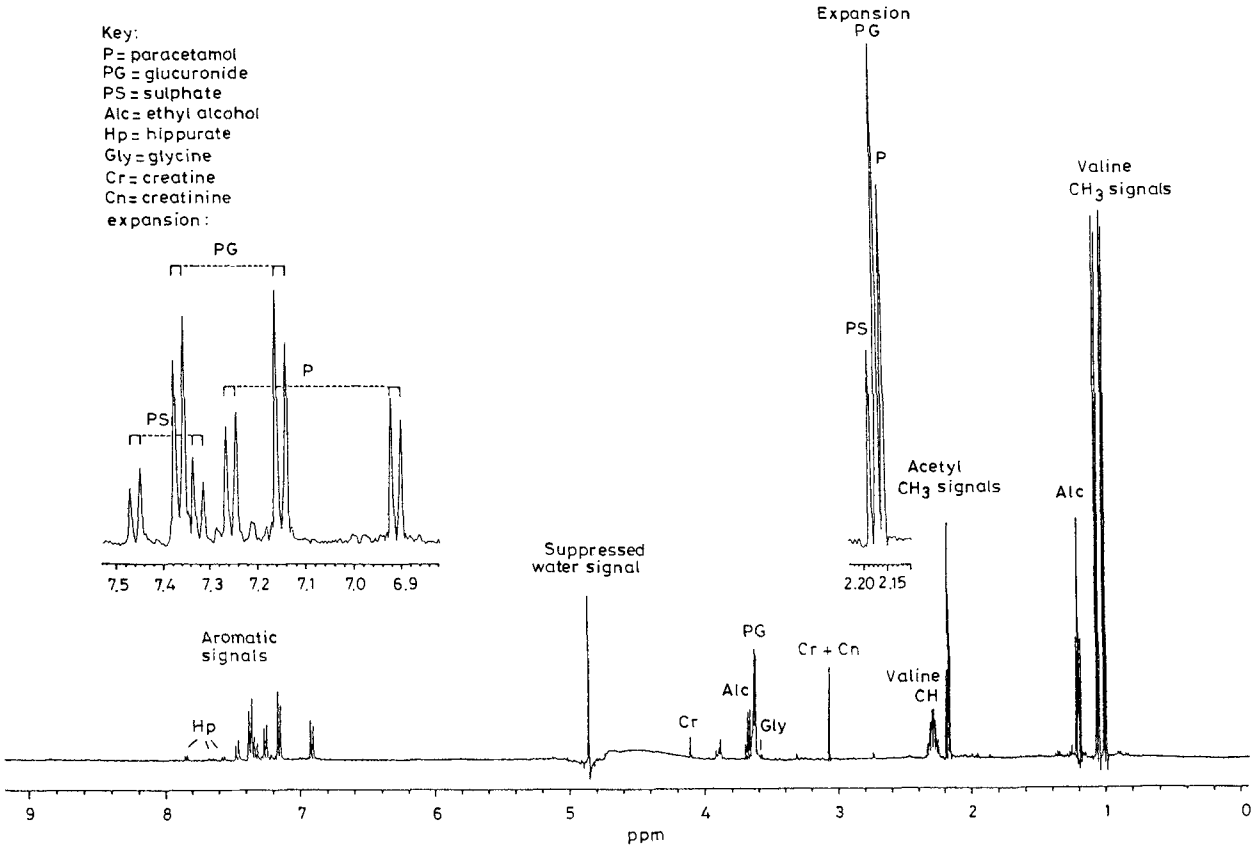


Figure 5.52. 400 MHz ^{1}H-NMR spectrum of urine from a patient who had taken an overdose of paracetamol. Valine was added as an internal standard.

naturally occurring as there are fewer background resonances in the spectrum of the biological fluid. In addition, the spectral widths of non-hydrogen nuclei are much wider and resonances less likely to overlap. Examples in this area include investigation of the metabolism of 5-fluorouracil [135], fluoropyrimidine [136, 137] and flucloxacillin [138] using ^{19}F-NMR.

In addition to the direct determination of drugs and metabolites in body fluids, it should be possible to obtain an indication of the overall biochemical changes associated with drug metabolism and toxicity by monitoring changes in the pattern of endogenous metabolites. This approach has been used to pinpoint the site of mercury-induced toxicity in rats [139] and to investigate the toxicity of the potential anticancer drug, *N*-methylformamide [140]. Studies of drug metabolism by *in vitro* cell suspensions or cell-free extracts have been done using various NMR-active nuclei. A ^{1}H-NMR investigation of paracetamol metabolism in liver hepatocytes and cell extracts showed that drug metabolites could not be observed in the intact cells but became apparent on lysis [141]. The incorporation of 5-fluorouracil into RNA and its conversion to metabolites in *Escherichia coli* cells has been followed by ^{19}F-NMR [142]. The metabolism of ^{13}C-labelled drugs by a perfused liver system has also been reported [143]. A review of NMR spectroscopy of biological samples as an aid to drug development has recently been published [143a].

SOLID-STATE CARBON-13 NMR SPECTROSCOPY

NMR spectroscopy is most commonly used to study molecules in solution, but great progress has been made in its application to solid-state systems [144–149]. In the solid state, molecules are held rigidly, so that dipolar interactions are not averaged to zero, as they are by motion in isotropic liquids, and lead to broad resonances. In addition, chemical shielding anisotropy (CSA) effects, due to the range of molecular orientations in the sample, contribute to a spread of frequencies for signals from solids. Together, these two effects lead to broad linewidths of several kHz for solids. Theoretically, they can be averaged out by spinning the sample at an angle of 54.7° (the so-called 'magic angle') to the applied magnetic field. However, the speeds required (tens of hertz) cannot be achieved in practice, so that the averaging process is not complete.

These problems have been overcome for dilute spins, that is, those of low natural abundance such as ^{13}C, by high-power decoupling to eliminate heteronuclear dipolar broadening. The technical difficulties due to the heating effects of the high-power input to the sample were overcome by pulsed decoupling

sequences. The remaining broadening due to CSA can be removed by magic-angle spinning (MAS) [150]. Poor sensitivity due to long relaxation times for ^{13}C is improved by cross-polarization (CP), whereby magnetization is transferred from ^{1}H nuclei to the carbon nuclei using the Hartmann-Hahn contact sequence, in which matching of the ^{1}H and ^{13}C frequencies in the rotating frame are achieved [151, 152]. CP-MAS spectroscopy is the combined application of these techniques [153]. Linewidths of only a few Hz can be obtained for powdered crystalline material, while signals from amorphous solids are broader. Chemical shifts are quoted with respect to TMS by using a secondary reference material and are comparable with those found in solution spectra. Pulse sequences for differentiation of multiplicities are available as in solution ^{13}C-NMR, one being used for non-quaternary suppression (NQS) [154].

The averaging of the larger homonuclear dipolar interactions which occur in proton spectroscopy is a more challenging problem. Multiple-pulse sequences are used together with MAS [155, 156], but residual linewidths are a few tens of hertz, so that resolution is not comparable to the solution state. Solid-state NMR of single crystals has been used to measure molecular geometry [157] and to gain understanding of the theoretical basis for chemical shifts [158] which are assigned empirically in solution spectra.

While solid-state NMR has found greatest application to the study of insoluble materials such as plastics, polysaccharides and zeolites, ^{13}C CP-MAS has also been used to study biomolecules such as amino acids and peptides [159–162], the steroids hydrocortisone [148] and testosterone [163] and ascorbates [164, 165]. Some applications to medicinal agents are described below. The relationship between chemical shift and molecular orientation leads to the splitting of signals where there is intra- or intermolecular non-equivalence, for example, where there is more than one conformer present or more than one molecule in the crystallographic asymmetric unit or where a substance can crystallize in more than one form. Polymorphic forms of pharmaceuticals, which may have different impacts on formulation and bioavailability, have been characterized by carbon-13 CP-MAS spectra [163, 166, 167]. Byrn, Gray, Pfeiffer and Frye [166] studied two polymorphs of benoxaprofen which had characteristic spectra and were able to use ^{13}CP-MAS as a qualitative method of determining whether a mixture of the two forms was present (see *Figure 5.53*). Quantitative analysis was not possible without careful optimization of experimental conditions taking into account proton relaxation effects. In addition, the form of benoxaprofen in a granulation mixture was identified, the excipients giving rise to signals which did not interfere with the spectrum of the pharmaceutical. In a study of aspirin, it was found that two crystalline forms gave the same spectrum, which

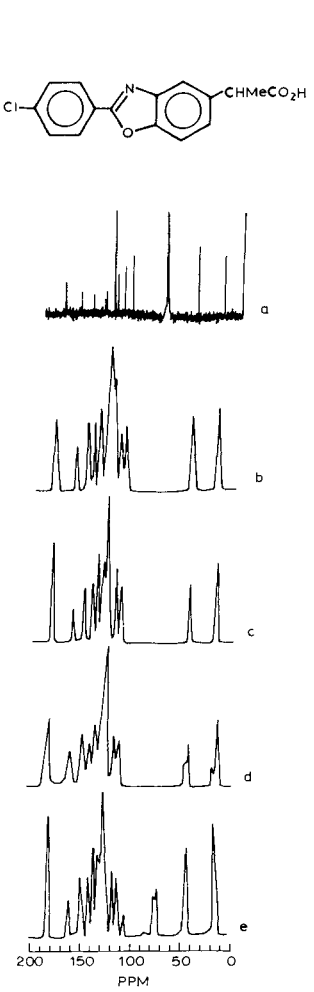
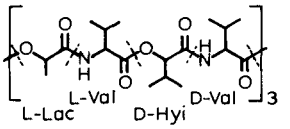


Figure 5.53



Valinomycin

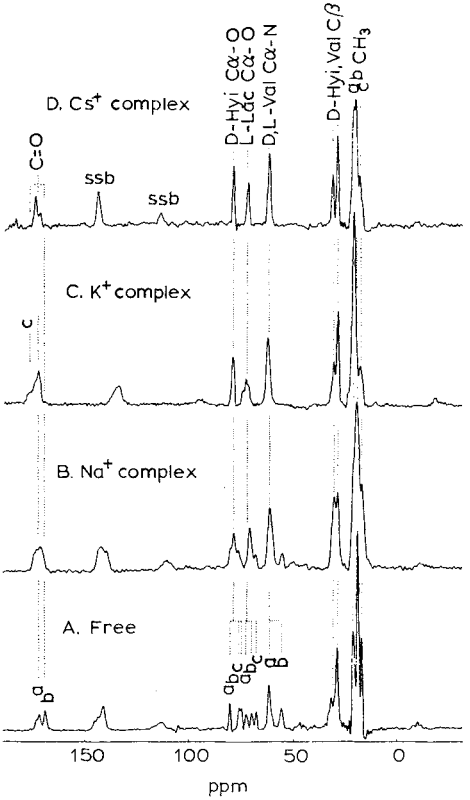


Figure 5.54

Figure 5.53. Solution and solid-state ^{13}C-NMR spectra of benoxaprofen. (a) Solution spectrum; (b) solid-state spectrum of form 1; (c) solid-state spectrum of form 11; (d) solid-state spectrum of a 1:1 mixture of forms 1 and 11; (e) solid-state spectrum of a granulation containing 66% benoxaprofen.

Figure 5.54. 75.46 MHz ^{13}C CP-MAS NMR spectrum of valinomycin and its complexes. (a) Free valinomycin; (b) NaSCN complex; (c) 0.5(KI_3 + KI_5) complex; (d) CsSCN complex.

suggested that the origin of the difference was not polymorphic [168]. In tablet formulations of aspirin, interactions with the buffer components were not observed unless the tablets were dissolved and lyophilized.

It should be possible to distinguish the crystals of pure optical isomers and racemic mixtures by solid-state NMR because intermolecular interactions render them diastereomeric whereas solute–solute interactions are generally so weak in solution that enantiomers and racemates have identical spectra. Hill, Zens and Jacobus [169] used solid-state ^{13}C-NMR to determine the (+)(*R,R*), (±) and *meso*-forms of tartaric acid. Each gave separate chemical shifts for the two carbonyl and two α-carbons in the molecule and the four chemical shifts were different for each form. Thus, the classical diastereoisomers could be distinguished and solid-state NMR forms the basis of a method of determination of optical purity.

Solid-state NMR may be useful for the investigation of conformational behaviour which is made difficult by rapidly interchanging forms in solution. Saitô has reviewed conformational characterization by solid-state ^{13}C-NMR which discusses mainly biopolymers [170]. An example of changes in conformation observed by ^{13}C CP-MAS spectroscopy is given by the solid-state spectra of free valinomycin and its complexes with Na^+, K^+ and Cs^+ [171]. In free valinomycin, a set of three peaks of equal intensity is observed for the C_α-O signals of the three DHyi and LLac residues, while the C_α-N signals of the three D- and LVal residues are split into two peaks of unequal intensities. On the other hand, the metal complexes showed similar spectra, corresponding to a single conformer, which were readily assigned by their similarity to the solution spectrum obtained in chloroform (*Figure 5.54*). These data were consistent with X-ray diffraction results which indicated that the three residues of each type of amino acid experience different local conformations in the free state but identical ones in the metal complex.

The valinomycin study just described illustrates the value of close comparison of NMR and X-ray diffraction data. Correlation of NMR assignments with X-ray data for known structures may allow the subsequent elucidation of conformational features for related materials on the basis of NMR data alone. It is notoriously difficult to relate solid-state structures determined by X-ray diffraction to the solution conformation observed by conventional NMR spectroscopy, although data from the former technique are often the only type available for work on receptor recognition. However, solid-state NMR may provide a link between these two techniques by indicating whether molecular conformation changes do occur when the physical state is altered. In contrast to the valinomycin complexes discussed above, it has been found that significant differences in the solution and solid-state spectra of morphine

[172, 173] were due to the effects of crystal packing forces and hydrogen bonding on the conformation of the molecule.

An extensive survey of the conformational states of crystalline penicillins has been carried out by Clayden, Dobson, Lian and Twyman [174]. Solid-state ^{13}C data were compared with solution spectra and X-ray crystal structures. The dynamic behaviour of the side-chain was evident in the spectra and depended on the crystalline environment of the molecules. In addition, two conformations of the thiazolidine ring could be identified by the chemical shifts of the geminal 2-methyl groups and enabled the estimation of the relative populations of these conformers in solution. *Figure 5.55* gives the solid-state spectra for penicillin salts adopting the two different conformations together with the solution spectrum of penicillin G showing the average chemical shifts obtained from the rapidly interchanging forms.

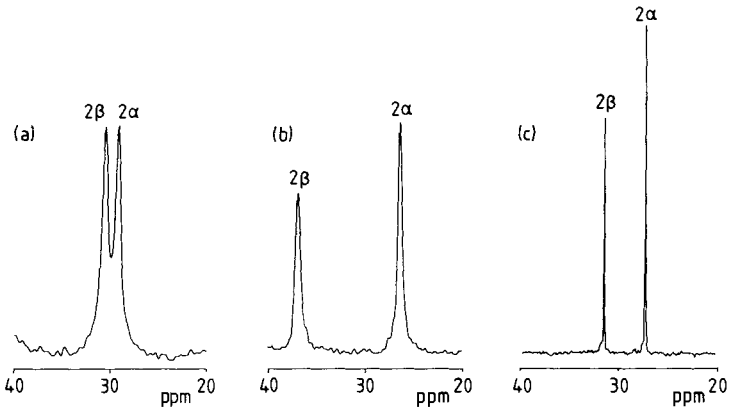


Figure 5.55. Solution and solid-state ^{13}C-NMR spectra of penicillin G. (a) 50.32 MHz CP-MAS spectrum of crystalline procaine penicillin G; (b) 50.32 MHz CP-MAS spectrum of crystalline potassium penicillin G; (c) 62.9 MHz solution spectrum of potassium penicillin G.

CONCLUDING REMARKS

In this review we have attempted to illustrate the power and versatility of modern high-field NMR techniques in providing information of a diverse nature relating to both man-made and natural materials. NMR methods are second to none in their ability to characterize molecular structure and geometry in the solute state, and are now finding increasing application to solid-state investigations. In addition to the intrinsic value of such work, data

so derived provide information fundamental to initiation of molecular modelling and computational procedures which are now well-established approaches to drug design and medicinal chemistry in general. Advances in the field allow routine analyses of relatively large molecules, a fact of special relevance to polymeric materials of biological importance. The non-invasive nature of NMR experiments coupled with the development of methods for the elimination of water and other solvent signals, make the technique ideal for examination of biofluids (including *in vivo* systems) and many novel applications of this kind are probable in the future.

Although examples used in this review are limited to protons, carbon-13 and a few other magnetic nuclei, it is important to emphasize the wide occurrence of atomic nuclei with magnetic properties, and that most elements occur in an isotopic form suitable for use as a magnetic probe [175]. To quote a few recent examples that concern the rarer magnetic nuclei, [^{77}Se]selenocysteine has been used to probe the active site of glutathione peroxidase [176], ^{2}H-NMR to study the phase behaviour of gramicidin-phosphatidylcholine mixtures [177], and thallium (^{203}Tl and ^{205}Tl) NMR to study the function of sodium and potassium cations in biochemical systems (for a review see Ref. [178]).

The technique of NMR imaging [179] falls outside the scope of this review, but should be mentioned in regard to its potential value in monitoring the pharmacokinetics of suitably labelled drugs, (for example, with ^{19}F) administered to small animals (and ultimately man) that may be accommodated within the NMR probe cavity. Further applications of dynamic NMR [180] to studies of interactions between small molecules and macromolecules (perhaps even pharmacological receptors and their ligands) are also anticipated.

If the growth of NMR technology maintains its present pace, it would take a brave person indeed to define its ultimate limits.

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6 Copper Complexes Offer a Physiological Approach to Treatment of Chronic Diseases

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INTRODUCTION

ESSENTIALITY OF COPPER

Copper is recognized as an essential metalloelement like sodium, potassium, magnesium, calcium, iron, zinc, chromium, vanadium and manganese [1]. Like essential amino acids, essential fatty acids and essential cofactors (vitamins), essential metalloelements are required for normal metabolic processes but cannot be synthesized *de novo* and daily dietary intake and absorption are required. The adult body contains between 1.4 mg (22 μmol) and 2.1 mg (33 μmol) of copper per kilogram of body weight, while the infant body contains 3-times this amount, consistent with the fact that infant metabolic

Table 6.1. MEAN TISSUE CONTENT OF COPPER FOUND IN TISSUES AND FLUIDS OF INDIVIDUALS WHO DIED A SUDDEN ACCIDENTAL DEATH [2,3]
Copper content is given as μg/g tissue ash or as shown.

Adrenal	210	Milk	
Aorta	97	colostrum	0.35–0.50 μg/ml
Bile	547	mature	0.20–0.50 μg/ml
Blood (total)	1.01 μg/ml	Muscle	85
erythrocytes	0.98 μg/ml	Nails	23 μg/g
plasma	1.12 μg/ml	Omentum	190
serum	1.19 μg/ml	Ovary	130
Bone	25 μg/g	Pancreas	150
Brain	370	Pancreatic fluid	105
Breast	6 μg/g	Placenta	4 μg/g
Cerebrospinal fluid	0.22 μg/g	Prostate	110
Diaphragm	150	Saliva	0.08 μg/ml
Esophagus	140	Skin	120
Gall bladder	750	Spleen	93
Hair	19 μg/g	Stomach	230
Heart	350	Sweat	0.55 μg/ml
Intestine		Testes	95
duodenum	300	Thymus	4 μg/g
jejunum	250	Thyroid	100
ileum	280	Tongue	4.6 μg/g
cecum	220	Tooth	
sigmoid colon	230	dentine	2 μg/g
rectum	180	enamel	10 μg/g
Kidney	270	Trachea	65
Larynx	59	Urinary bladder	120
Liver	680	Urine	0.04 μg/ml
Lung	130	Uterus	110
Lymph node	60		

needs are that much greater than those of adults. All body tissues need copper for normal metabolism, but some tissues have greater metabolic needs than others and tissue content of copper reflects this fact. Amounts of copper found in various body tissues and fluids of individuals [2,3] are shown in *Table 6.1.*

The amount of copper in each tissue correlates with the number and kind of metabolic processes requiring copper in that tissue. In this regard, it is of interest to point out that brain and heart contain more copper, about 360 μg/g of tissue ash, than all other tissues except the liver, which contains about 700 μg/g of tissue ash and functions as a major copper storage organ. Gall bladder and bile also contain a large amount of copper, 550 to 750 μg/g of tissue ash, which has been attributed to their suggested role in excretion [1]. However, the gall bladder may also serve as a storage tissue and bile may contain a mobile storage form of copper suitable for intestinal reabsorption, as evidenced by the presence of low-molecular-weight complexed forms in bile [4]. The large kidney copper content, 270 μg/g of tissue ash, when compared with the very small urine copper content, 0.04 μg/ml, suggests a conservatory role for the kidney. Gastric, intestinal and adrenal tissues also have high copper content, 200 to 300 μg/g of tissue ash, and this reflects their high metabolic rates. Remaining tissues have lesser amounts of copper because of their relatively lower metabolic activity, but it is just as important for normal metabolism in these tissues as it is in all others.

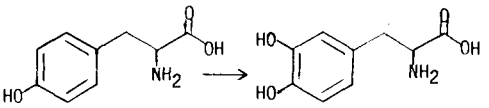
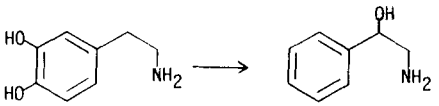
Although bile may serve as the major excretory vehicle for excess copper, significant but lesser amounts of copper are lost via hair, stratum corneum, finger- and toe-nails, sweat and urine as end-products of metabolism. These losses point out the need for compensating daily intake and absorption to replenish this essential metalloelement.

Ionic copper has a particularly high affinity for other molecules (ligands,L) capable of bonding with it. A consequence of this is that all measurable copper in biological systems exists as complexes or chelates composed of copper bonded to organic components of these systems. Calculated amounts of ionic copper suggested to be present in biological systems (10^{-18} M in plasma) [5] are too small to be measured using the most sensitive instrumentation available. As a result, measurable tissue copper content reflects content of copper complexes and these complexes account for the absorption, distribution and biologically active forms of copper *in vivo.*

COPPER-DEPENDENT MAMMALIAN ENZYMES

Recognized and recently suggested copper-dependent mammalian enzymes [6–19] are listed in *Table 6.2.* Cytochrome *c* oxidase is required by all cells

Table 6.2. COPPER-DEPENDENT MAMMALIAN ENZYMES AND THEIR CHEMICAL FUNCTION [6–19]

Enzyme	*Function*
Cytochrome *c* oxidase	reduction of oxygen: $O_2 \xrightarrow{H^+,e^-} HO_2 \xrightarrow{H^+,e^-} H_2O_2 \xrightarrow{H^+,e^-} H_2O + HO^{\bullet} \xrightarrow{H^+,e^-} H_2O$
Superoxide dismutase	disproportionation of superoxide in prevention of its accumulation: $2O_2^- + 2H^+ \longrightarrow O_2 + H_2O_2$
Tyrosinase	Hydroxylation of tyrosine in melanin synthesis: 
Dopamine-β-hydroxylase and extremely acidic copper-containing protein	hydroxylation of dopamine in catecholamine synthesis: 
Lysyl oxidase	oxidation of terminal amino group of lysyl amino acids in procollagen and proelastin to an aldehyde group: $\text{peptidyl}(CH_2)_3CH_2\text{-}NH_2 \longrightarrow \text{peptidyl-}(CH_2)_3\text{-}CH{=}O$
Amine oxidases	oxidation of primary amines to aldehydes in catecholamine and other primary amine metabolism: $R\text{-}CH_2\text{-}NH_2 \longrightarrow R\text{-}C{=}O$
Ceruloplasmin	mobilization and utilization of stored iron: ferroxidase Fe(II) $\longrightarrow$ Fe(III) copper transport, SOD-like activity, serum amine oxidase activity and angiogenic activity
Factor V	blood clotting
Peptidyl α-amidating mono-oxygenases	synthesis of neuroendocrine peptides (hypothalamic thyrotropin releasing hormone, α-melanocyte stimulating hormone from anterior pituitary, oxytocin and vasopressin from the posterior pituitary, gastrin from stomach and choleocystokinin from the small intestine): $\text{peptidyl-N}(H)\text{-}CH_2C(=O)OH \xrightarrow[\text{vit C}]{} \text{peptidyl-}NH_2 + HC(=O)C(=O)OH$

to produce energy needed to drive energy-requiring biochemical reactions. Cytosolic [9, 10] and extracellular superoxide dismutases (SODs) [13, 14] are required to prevent cellular destruction associated with accumulation of superoxide (O_2-) and other oxygen radicals (HOO, HO, $^1O_2$) derived from it. There are many suggestions concerning roles these oxygen radicals play in causing pathologic changes associated with affected tissues in chronic diseases [15]. Tyrosinase is the enzyme required for synthesis of dihydroxyphenylalanine (DOPA) and its subsequent transformations to melanin, required for pigmentation, as well as a possible source of DOPA for catecholamine synthesis. Dopamine-β-hydroxylase and the extremely acidic copper-containing protein are required for conversion of dopamine to norepinephrine (noradrenaline) [6], which is then converted to epinephrine (adrenaline). Lysyl oxidase is required for cross-linking of collagen and elastin in maintenance and repair of all connective tissues [16]. Amine oxidases are required for removal of primary amines which are no longer needed for hormonal activity, that is, norepinephrine or dopamine or other amines which are toxic metabolic end-products. Ceruloplasmin, which represents 70–90% of the copper contained in plasma components, is now suggested to be multifunctional, having transport function, serum antioxidant (SOD-like) activity,serum amine oxidase activity, ferroxidase activity in mobilizing stored iron [Fe(II)] for haemoglobin synthesis [7], and angiogenic activity required for vascularization [17]. Clotting factor V is also suggested to be copper-dependent [18]. Peptidyl α-amidating monooxygenases are a newly recognized class of copper-dependent enzymes suggested to be required for syntheses of neuroendocrine peptides, including α-melanocyte-stimulating hormone, thyrotropin-releasing hormone, oxytocin, vasopressin, gastrin and cholecystokinin from their precursor glycyl-terminus peptides [19].

INGESTION, ABSORPTION, DISTRIBUTION, UTILIZATION AND EXCRETION OF COPPER COMPLEXES

Ingested copper complexes representing the recommended 3 mg (47 μmol) daily intake of copper follow the pathway presented in *Figure 6.1*. One (CuL_2) of a large number of possible copper complexes that might be found in foods and/or beverages, following ingestion and digestion, would give rise to the formation of other bis or mixed binary copper complexes as a result of exchange with ligands (L: amino acids, fatty acids, amines, etc.) in the enzyme digest or ternary complexes of the original complex. Ternary complexes are complexes formed by the addition of another ligand, such as a small peptide or amino acid, to an existing complex to form a new complex having a larger

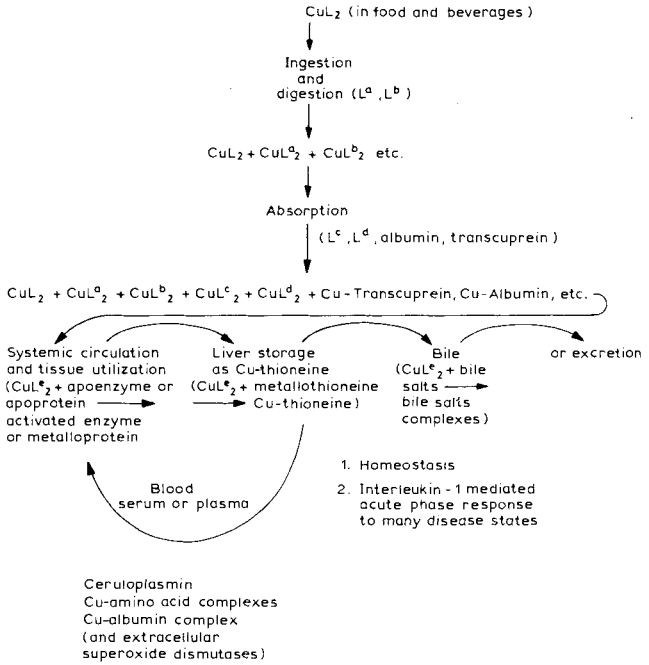


Figure 6.1. Ingestion, absorption, distribution, utilization, and excretion of copper complexes.

molecular mass. Gastric digestion is enzyme-catalyzed and not acid-catalyzed and, since the gastric pH is likely to range from 6 to 3 with the ingestion of a meal [20], as shown in *Figure 6.2*, some of the originally ingested complex may be absorbed intact. Copper complexes in the duodenal chyme (pH 7.0)

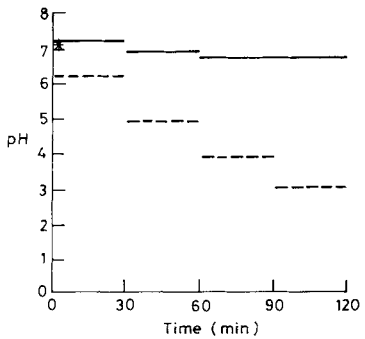


Figure 6.2. Changes in the pH value of chyme entering the duodenum (- - - -) and leaving the duodenum (———) following the ingestion of a pH 7.1-meal(*) [20], as adapted from Davenport [21].

would also be expected to be absorbed intact. Pharmacologic doses of copper complexes have an antisecretory effect [22] and thus prevent lowering of the normal empty stomach pH (6.0).

Additional complexes such as Cu-transcuprein [23[(*Figure 6.1*), which may be a ternary complex of an absorbed smaller complex, or a ternary albumin complex may also be formed, without ligand exchange, following systemic absorption. A Cu-albumin complex has been suggested to have extracellular storage function [24]. Again, depending upon the concentration absorbed, some of the original complex may remain intact in plasma. All of these copper complexes then undergo systemic circulation to all tissues and are (1) utilized by tissues following ligand exchange with apoenzymes and apoproteins to form metalloenzymes and metalloproteins, (2) stored in the liver following ligand exchange with thioneine to form copper(I)-thioneine, or (3) excreted in the event that tissue needs have been met and stores replenished. Since excessive copper storage has never been reported in any normal population, chronic copper poisoning has not been described in normal humans [25], and copper complex absorption and excretion must vary from small to relatively large quantities in these populations, efficient homeostatic mechanisms must regulate retention and excretion of varying amounts of absorbed copper complexes.

Also shown in *Figure 6.1*, under homeostatic controlled conditions copper(I)-thioneine-stored copper is released from the liver in a circadian fashion [26] as complexed forms, ceruloplasmin [27], copper amino-acid complexes, and a copper albumin complex, to meet normal metabolic needs. This homeostatic release of copper complexes from the liver meets normal copper-dependent physiologic requirements of body tissues, which includes *de novo* synthesis of copper-dependent enzymes, provided stores are adequate.

The essentiality of copper is now understood as being based upon its recognized need for activation of copper-dependent enzymes. Complexed forms of copper also facilitate absorption, tissue distribution and tissue utilization. In the non-disease state, these forms of copper account for the physiologic regulation of copper-dependent homeostatic processes. Since copper is needed for normal metabolism and prevention of disease, great care should be taken to assure that dietary intake provides required amounts of copper. Unfortunately, a review of studied modern diets leads to the conclusion that many or nearly all diets do not supply required amounts of this and other essential metalloelements [27, 28]. It is then reasonable to suggest that marginal or deficient intakes or impaired absorption may lead to decreased copper-dependent enzyme activity and manifestations of acute disease in the short term as well as manifestations of chronic disease with long-term decreases in copper-depedent enzyme activity.

INTERLEUKIN-1-MEDIATED INCREASE IN PLASMA COPPER COMPLEXES

As is also shown in *Figure 6.1*, an interleukin-1-mediated [29] acute-phase response to many diseases involves a release of copper-thioneine-stored copper from the liver as ceruloplasmin, copper amino-acid complexes, and a copper albumin complex to meet increased metabolic needs for copper, which exceed normal needs, and plasma copper concentrations increase 200–300% above normal, as illustrated in *Figure 6.3*. The appropriate increase coupled

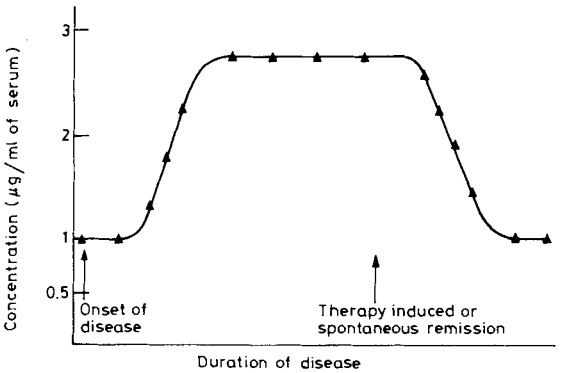


Figure 6.3. Alterations of serum copper content in the general interleukin-1-mediated acute phase response to diseases.

with an ability to sustain this response may lead to a spontaneous or facilitated therapy-induced remission of disease within a relatively short period. However, when liver stores are inadequate and this copper-dependent response cannot be maintained, remission cannot occur, and the disease persists as chronic disease.

INFLAMMATORY DISEASES

ALTERED COPPER METABOLISM IN ARTHRITIC AND OTHER DEGENERATIVE DISEASES

Altered copper metabolism in rheumatoid arthritis (RA), osteoarthritis, and other chronic degenerative diseases, which are also historically known as connective tissue diseases because of associated pathological changes involving these tissues, has been an area of active research interest [30] since Heilmeyer and Stuwe [31] originally reported elevated plasma copper levels in RA patients.

In spite of an abundance of literature, there is still no singularly accepted interpretation of results reported to date. The earliest reported alterations were interpreted as physiologic changes which checked the disease processes [31], while subsequent results were interpreted as pathologic changes of the disease processes [32]. However, relatively recent progress in essential metalloelement research has provided evidence which supports the earlier interpretation that observed alterations are physiological rather than pathological, although this latter notion still persists [33] based upon results obtained with inappropriate additions of copper or iron salts and vitamin C or hydrogen peroxide in non-physiological *in vitro* studies [34–38]. Recent symposia [39, 40] and reviews [41–45] lend support to the notion that the elevation in plasma copper represents a physiological facilitation of remission, when remission results, or the less than required elevation as a failure of the physiological response resulting in chronic complicated disease.

Studied variations of copper in tissues of individuals with RA have been published for the last 50 years. Heilmeyer and Stuwe were first to report a total plasma copper increase in men and women with active RA which returned to normal with remission [31]. The increase in copper was observed to occur so regularly in plasma or serum that it was thought of as a law with great prognostic value and preferable to routine erythrocyte sedimentation rate (ESR) determinations [45]. It now seems reasonable to suggest that the synthesis and secretion of ceruloplasmin from the liver and release of lower-molecular-weight copper complexes from the liver represent copper-containing acute phase reactants [46–48]. This provides a rationale for the observed changes in copper-containing components in blood as sequential disease-checking processes.

These changes in serum copper were found not to be related to sex but were most pronounced in the fever phase and correlated with a decrease in haemoglobin and an increase in ESR [45]. Non-sex-related alterations of copper were confirmed by Van Ravesteijn, who also found that RBC copper decreased in spite of an increase in whole blood copper [49].

Subsequently, Brendstrup pointed out that the increase in serum copper in febrile patients returned to normal in remission [50, 51]. Of perhaps even greater significance was that the change in copper was shown to be directly related to the activity or severity of RA. Active disease was associated with increased serum copper which was directly related to decreased mobility and haemoglobin values as well as increased ESR and duration. Mean serum copper value for patients with normal mobility differed relatively little from those for patients with inactive joint lesions. Patients with active RA who showed clinical improvement had a pronounced fall toward normal serum

copper and ESR, while these parameters remained relatively unchanged in patients showing no improvement. These results were supported by subsequent findings for Grade IV [52] (American Rheumatism Classification [53]) RA patients with morning stiffness, diminished hand grip, and hand joint inflammation, with more than two involved joints or, if only two were involved, displaying marked involvement, including large effusions and fever.

Brendstrip's data also confirmed that normal sex-related serologic difference in serum copper was no longer apparent in the active disease state because of marked increases in concentration for both men and women. In addition, these serologic changes were most pronounced in patients with the most active rheumatic disease. These results led Brendstrup to suggest that the degree of activity of the pathological process or disease could be evaluated by determining the magnitude of serum copper alteration. Alternatively, the rate of remission was related to the rate of return to normal concentration.

Results published from 1938 to 1953 can be viewed as having clearly demonstrated that patients with RA had a higher mean serum copper concentration than do normal healthy individuals. Small sex-related differences in normal individuals were obscured by marked increases found for both male and female patients. The increase in serum copper associated with the onset and persistence of active disease returned to normal with disease remission. It is now known from animal studies [46, 47] that the rise in serum copper is accompanied by a fall in total serum zinc and iron. This fall in total serum zinc and iron is partially accounted for by their requirement for the synthesis of copper-containing components in the liver.

Changes in serum copper were found to be directly related to disease severity or activity as measured by increased corporal and local temperatures, immobility, duration of disease, pain, oedema and ESR, as well as diminished strength and decreased haemoglobin value. Changes in serum copper were also found to be most pronounced in very active RA, less pronounced in less active disease, and normal in remission or inactive disease.

The acute phase determinant, copper, was viewed as having more diagnostic and prognostic value than ESR or other acute-phase reactants. Acute-phase changes in serum copper were suggested to be associated with disease-checking processes. This suggestion is consistent with the notion that changes in serum copper represent a part of the multifaceted physiological response to inflammatory diseases [30, 43, 44, 48, 54–63].

A low or nearly normal serum copper value may be the result of a failure of this aspect of the physiological response due to depletion of liver copper stores. Depletion may result from increased turnover resulting in increased copper excretion losses [32, 64–68] and a failure to replete these stores either

through loss of appetite or inadequate dietary intake, which is a likely possibility associated with modern food processing. It has recently been suggested that no more than 25% of the United States population has an adequate daily dietary copper intake [69]. Failure of this aspect of the physiological response for these reasons could lead to chronic disease.

Following reports by Brendstrup in 1953 [50, 51], there was little published concerning concentration changes in blood-essential metalloelements associated with RA until 1965, when interest in this topic was renewed. In 1965 Plantin and Strandberg [70] reported results which confirmed the report by Van Ravesteijn [49] that total whole blood copper was elevated in RA. Analyses of whole blood for copper gave values which were essentially the same regardless of disease activity. Thus, analysis of whole blood for copper may not provide useful diagnostic or prognostic information concerning the activity of this disease.

Earlier observations concerning copper in RA were extended with the report that the increase in serum copper was due to an increase in ceruloplasmin (Cp) and non-Cp copper concentrations [71]. The observed mean increase in Cp represented an increase of 34 μg/100 ml, while the mean non-Cp copper increased only 11 μg/100 ml of serum.

Increased synthesis of Cp in response to RA was subsequently confirmed and shown to be related to disease activity [33, 65–66, 72–74]. Patients with severe to moderately active RA had significantly ($P < 0.05$) accelerated daily Cp turnover rates [65]. The highest turnover rate was found for a patient with the highest Cp concentration and severely active RA. Moderately active RA was associated with lower concentrations of Cp and moderate turnover rates. These results indicated that increased daily turnover was accompanied by increased rate of synthesis and, in the steady rate, a corresponding increase in elimination rate [66]. Gamma-globulin turnover studies also revealed an accelerated synthesis of this protein in the majority of patients with RA [66].

Lorber, Cutler and Chang [75] were the first to report a statistically significant elevation of serum copper concentration in RA when compared with that in age-matched normal adults. (A recent comparison of age-matched children demonstrated that serum copper is elevated in patients with juvenile rheumatoid arthritis [76].) They also reported that almost the entire serum copper content of normal individuals was bound to Cp [75]. This was corroborated in a second group of normal individuals with the demonstration that serum copper contained only a small amount of non-Cp copper (7 μg/100 mg), which was somewhat smaller than reported by others (24 $\pm$ 16 μg/100 ml) [71]. In addition, their population of RA patients had a very large non-Cp copper concentration (103 μg/100 ml) [77], which was much larger than re-

ported by others (35 ± 25 μg/100 ml) [71] or (9 μg/100 ml) [78]. A significant ($P < 0.001$) positive correlation between increase in non-Cp bonded serum copper content and disease activity was confirmed [72] and extended by a number of other research groups [57, 73, 79]. The suggestion that the less than normal amounts of albumin and low-molecular-weight forms of copper found in plasma of RA patients and higher than normal amounts of total and Cp bonded copper indicate a lack of copper bioavailability [76] is in contrast to the suggestion of others that Cp is a bioavailable transport form of copper [59]. Based upon these observations, it seems reasonable to interpret the large increase in non-Cp copper as an integral part of the physiological response to active disease in these patients.

It may be possible to accommodate these observed differences by taking into account disease activity and the response to disease in these three populations. The second group of RA patients [75] seem to have had the most active disease or were more able to respond to their disease, as these patients had the highest serum copper concentration (248 μg/100 ml). Since patients in the first [71] and third [78] groups had serum copper concentrations of 192 and 150 μg/100 ml, respectively, these patients may have had less active disease, were in remission or were no longer physiologically responding to their disease as a result of depleted liver stores, which may have been the situation in a fourth group of patients [85] who had essentially normal serum copper levels.

It was concluded [75] that the elevation in serum copper associated with RA was due primarily to an elevation of non-Cp copper and it was suggested that this copper might serve as a deleterious sulphydryl group oxidizing agent; denaturing haemoglobin and disrupting RBC and lysosomal membranes, resulting in the release of tissue lytic enzymes. The increase in Cp and decrease in serum thiol level was confirmed [63, 74] and it was demonstrated that haemolysate superoxide dismutase (Cu-Zn SOD) activity decreased while the thiol level increased, reflecting additional changes in oxidative status which may have further implications concerning pathogenesis (disease activity) of RA (duration of morning stiffness, pain score, grip strength, articular index, ESR and haemoglobin values). Comparison of copper-dependent SOD and Cp levels with cellular and plasma thiol levels in gold sodium thiolmalate- and auranofin-treated RA patients revealed changes indicative of beneficial therapeutic effects in those patients that improved with therapy [63]. Beneficial effects of certain chelating agents such as penicillamine were attributed to their copper-chelating action, which presumably was believed to promote the excretion of copper [75]. This was used to account for beneficial effects of penicillamine resulting in lowered serum copper, increased serum

sulphydryl content, and decreased rheumatoid factor titres in patients receiving penicillamine. However, the observation [80] that copper stabilizes lysosomal membranes, perhaps via sulphydryl group oxidation, and as a result decreases the ratio of free versus bonded lysosomal enzymes, contradicts this interpretation [80]. Also, the mechanism of action of penicillamine and other chelating drugs which appear to lower serum copper by bringing about remission has been suggested to be the result of copper-drug complex formation, or mobilization of stored copper in the case of treatment with gold compounds, *in vivo*, which facilitates remission by promoting tissue utilization of copper rather than by promoting excretion [81–84]. Based upon these considerations, it does seem reasonable to interpret the large increase in non-Cp copper as an integral part of the physiological response to active disease which facilitates remission.

The well-known sex difference (female to male ratio 2:1) in populations of arthritic patients was suggested to account for elevated levels of copper and Cp due to an oestrogen-mediated increase in plasma copper concentration in the female subgroup of RA populations [86]. However, published data did not show that female RA patients, who were not known to be receiving oestrogen therapy, had a significantly different level of serum copper or Cp when compared with that of male RA patients. On the other hand, other data did show that the increased mean serum copper ($199 \pm 16\ \mu g/100\ ml$) and Cp ($50 \pm 7\ \mu g/100\ ml$) values found for normal females given oestrogen were slightly higher than mean values obtained for male RA patients and female RA patients who were not receiving oestrogen therapy [67]. This oestrogen-induced elevation in serum copper was still not as great as the elevation in serum copper found for patients with very active disease [51, 87, 88].

Many studies have provided information concerning serum copper and Cp levels found in RA patients receiving various anti-arthritic drugs [54–56, 60, 63, 72, 79, 89]. The mean copper and Cp concentrations found in female patients treated with intra-articular corticoids or treatment with ACTH were significantly ($P < 0.001$) higher than the values found in the female control group, but lower ($P < 0.001$) than the values obtained for female patients treated with non-steroidal anti-arthritic drugs (NSAAD), such as salicylates, indomethacin, ibuprofen or phenylbutazone [54]. Serum copper and Cp levels reported for male and female patients treated with aurothiomalate (chrysotherapy) was significantly ($P < 0.001$) higher than normal but significantly lower than the value found for patients treated with NSAAD [54, 63]. Lowest concentrations of serum copper and Cp were found for patients treated for 6 months with penicillamine. Interactions between penicillamine and copper and consequences associated with the use of penicillamine to treat RA have

been broadly considered [60, 72, 90, 91]. Although disease activity is uncertain in most of these patient populations, these observations are of special interest since copper complexes of NSAAD, corticoids, penicillamine and other complexing agents have been found to have potent anti-inflammatory activity and *antiulcer* activity [83, 84, 91, 92]. Based upon these observations, it was suggested that copper complexes, formed *in vivo*, were the active forms of these drugs [83]. This suggestion was supported and extended with the report that copper complexes are more potent analgesics than their parent ligands [93]. These observations are also consistent with the therapeutic effectiveness of copper complexes in the treatment of rheumatoid and other degenerative diseases [94]. Both the anti-inflammatory activity and therapeutic effectiveness of copper complexes as well as the pro-inflammatory and immunocompromising effect of copper deficiency support the suggestion that the rise in serum copper and Cp levels in RA is a physiological response which may facilitate remission [58, 95–101]. The observation that copper complexes are more effective as anti-inflammatory and analgesic agents and that they possess potent antiulcer activity, unlike existing drugs which are ulcerogenic, supports their use to treat rheumatoid and other degenerative diseases rather than searching for complexing agents designed to strip copper *in vivo* from those endogenous chelates that would appear to be devoid of pharmacological activity [58, 100].

It was also reported [54] that male and female RA patients had higher ($P < 0.001$) than normal mean serum antioxidant activity. The mean antioxidant activity obtained for 100 normal individuals (68 $\pm$ 10% inhibition) was significantly ($P < 0.001$) less than the value obtained for 100 RA patients (81 $\pm$9% inhibition). Female patients treated with NSAAD and chrysotherapy had higher antioxidant activities than the corresponding male population. A significant ($P < 0.001$) correlation between Cp and serum antioxidant activity was suggested to support the possibility that Cp was the dominant antioxidant. The antioxidant activity of Cp was suggested to play a protective role in situations involving tissue destruction and an increase in activity was suggested to be an important component of the systemic response to inflammation. It was pointed out that there was some evidence for a correlation between serum copper and the extent of erosive damage as seen in graded radiographs. In addition, the antioxidant activity may in part represent an increase in superoxide dismutase mimetic activity which has been found for low-molecular-weight copper complexes [44] and Cp [102]. These may also play an important role in the systemic anti-inflammatory response [62, 63]. The exact relationships between elevated antioxidant activity, serum copper, and Cp levels as well as tissue selenium content [61, 63, 89, 103] may represent

the induction of SOD and glutathione peroxidase activities in tissues undergoing oxidant stress.

Data have also been collected to show concentration changes for copper in synovial fluid (SF) and synovia of RA patients. Following the qualitative observation that Cp was present in SF [104], its presence and alteration in RA [71, 85, 105] and osteoarthritis [61] was quantitated. A significant increase in total SF copper was shown to be due to a large increase in SF Cp while the non-Cp copper concentration decreased. There was no obvious correlation between serum copper and Cp concentrations in SF and functional status, clinical stage, or medication which patients received at the time of SF aspiration, though the increased Cp concentration in SF appeared to approximately parallel duration of disease [85]. It is now possible to understand the increase in SF copper as a physiological response to prevent hyaluronic acid depolymerization and the destruction of other tissue components due to the accumulation of superoxide and other oxygen-radicals [106–109].

In addition to an increase in serum copper and total SF copper, SF from RA patients was found to contain two copper-containing components as compared with only one in SF from non-inflamed joints [110]. Ceruloplasmin was identified in all SF and the other component, found only in inflamed joints, was suggested to be a copper-albumin complex, although it may be extracellular SOD [109].

Copper, Cp and transferrin, as well as total protein and albumin concentrations in SF, were compared with those found in serum from patients with RA or osteoarthritis [111]. Mean serum copper and Cp values obtained for the RA group were significantly ($P < 0.001$) greater than the mean obtained for the group with osteoarthritis. Both groups had positive correlations for increases of SF copper and SF Cp. However, the SF non-Cp copper content was 22.5 ± 0.6 μg/100 ml for the osteoarthritic group, but none was found for the group with RA. Mean SF total protein for the group with RA was significantly ($P < 0.02$) higher than that obtained for the osteoarthritic group. Mean levels of SF albumin and transferrin were not significantly different for these two groups. Comparison of the SF:serum ratios of total protein, 0.55; albumin, 0.61; transferrin, 0.72; and Cp,0.69 in the RA group with the corresponding values for the osteoarthritic group, 0.46, 0.64, 0.63 and 0.42, demonstrated that effusion composition was different in these two diseases [54]. The rise in SF Cp was again suggested to be a protective acute response to inflammation because of its antioxidant activity.

An attempt to demonstrate the presence of copper in rheumatoid synovia using histochemical methods was unsuccessful [112]. It is questioned as to

whether or not these histochemical techniques are useful in this regard, since the synovia investigated were known to contain large quantities of iron, which may have interfered with histochemical determinations of copper.

In summary, reported results contribute to an understanding of altered copper metabolism associated with RA and osteoarthritis and its patterning in these and other degenerative diseases [113]. Marked increases in serum copper were confirmed in patients with early active disease. The observed alteration in serum copper is now understood as being mediated by interleukin-1 [29]. The increased rate of synthesis and accelerated turnover-rate of Cp was found to be directly related to disease activity. Synovial fluid copper content was found to increase in RA and osteoarthritis. Ceruloplasmin, which accounted for most of the SF copper increase, was found to increase with increasing duration of disease.

Marked increases in blood copper containing components, accelerated rates of Cp synthesis and turnover, and accumulation of low-molecular-weight copper-containing compounds and Cp in SF and synovial membranes may reflect changes in copper-requiring biochemical processes which can be interpreted as a component of the multifaceted physiological response to inflammatory disease and correlated with disease activity, duration, severity, pain, ESR, Hb levels, oedema, grip strength and immobility to achieve a better understanding of inflammatory diseases and devise better approaches to therapy.

ALTERED COPPER METABOLISM IN ANIMAL MODELS OF INFLAMMATION

Serum copper content, Cp and albumin and amino-acid copper complexes are also known to increase in animals in response to irritants [1, 114, 115], infections [31, 45, 116, 117], as well as in recognized animal models of inflammation [31, 45, 116–120], including rat polyarthritis, and the inflammatory response was found to be more severe in animals with established copper deficiency [121–125]. This impaired inflammatory response was related to altered immunocompetency [125] associated with copper deficiency which appears to be made worse with the consumption of diet with a high content of reducing sugars or carbohydrate that yields reducing sugars when digested [126].

Detailed studies of the concentration of copper and Cp in serum from rats with carrageenan foot-oedema and in serum and exudate from rats with carrageenan pleurisy revealed increases in serum copper and Cp in both models of inflammation [127, 128]. The increase in serum copper and Cp in the pleurisy model was strongly correlated with their increase in the exudate

[127, 128] and total liver copper content increased along with an increase in liver weight [128].

A study of the behaviour of haptoglobin, another acute-phase protein, and serum copper in adjuvant arthritic rats treated with ibuprofen demonstrated that increased haptoglobin and serum copper correlated with exacerbation of adjuvant arthritis [129]. Serum copper level (136 μg/100 ml) peaked at day 21 and haptoglobin peaked at day 28 when ibuprofen 25 mg/kg was given orally once a day for 4 weeks. With 75 mg/kg, inflammation was further reduced and serum copper again peaked at day 21, but this peak concentration (111 μg/100 ml) was not markedly elevated above the initial concentration (100 μg/100 ml) before returning to normal by day 28. Haptoglobin peaked on day 21 and began declining by day 28. Both treatment groups had decreased serum copper and haptoglobulin concentrations when compared to nontreated control adjuvant arthritic animals at all time periods during the course of treatment and these decreases were dose-related. The level of haptoglobin and serum copper clearly correlated with the intensity of adjuvant arthritis.

Anti-arthritic medicinal substances obtained from plants and hydrocortisone were studied with regard to their effect on essential metalloelement metabolism in the adjuvant-arthritic rat following oral treatment for 15 days. Medicinal substances derived from *Withania somnifera* and *Clerodendron inerme* decreased copper levels on day 3 compared with nontreated control levels, while hydrocortisone (15 mg/kg) had no effect. However, hydrocortisone treatment lowered serum copper values on days 15 and 49 after induction of inflammation, while the plant-derived drugs did not lower serum copper values [130].

The influence of oxyphenbutazone and hydrocortisone on Cp levels was also studied in various models of inflammation [131]. Ceruloplasmin was found to be 2-times the normal value in serum of carrageenan paw oedema, cotton wad granuloma, adjuvant arthritis and granuloma pouch models of inflammation, and this increase was suggested to be one of the body's inbuilt defence mechanisms against inflammation. Injection or oral administration of 80 mg/kg oxyphenbutazone or 20 mg/kg hydrocortisone did not lower Cp levels in the carrageenan, granuloma pouch, cotton wad granuloma and adjuvant arthritis during the course of treatment. Adjuvant arthritic animals were treated for 10 days beginning on day 20 post-adjuvant injection (established adjuvant arthritis). The granuloma pouch model was also treated for 10 days beginning 24 h after croton oil injection. Cotton wad implanted rats were treated for 7 days beginning 1 day after implantation. Rats injected with carrageenan were treated orally 1 h prior to carrageenan injection [131]. Failure to cause a decrease in Cp with these treatments suggests that longer

treatment may be required to decrease inflammation in each of these models. Examined changes in Cp, which was suggested to be a non-exchangeable form of copper, and reversibily bound forms of copper, albumin and low-molecular-weight complexes, measured in plasma from day 0 to day 20 demonstrated that, while total copper and Cp copper increased initially, the bioavailable form decreased progressively prior to the appearance of adjuvant arthritis. These results were offered as a rationale for the paradox of elevated plasma copper in RA and the antiarthritic properties of copper compounds [132].

ANTI-INFLAMMATORY ACTIVITIES OF COPPER COMPLEXES

Continued interest in copper complexes as anti-inflammatory agents and their potential as antiarthritic drugs is evidenced by the number of reviews and symposia proceedings published recently [22, 39–44, 133–139]. This selection contains both an historical and an updated review of studies of copper metal, inorganic compounds, and complexes as anti-inflammatory agents.

Before 1969, occasional publications reported that sodium 3-(*N*-allylcuprothiouredo)-1-benzoate [140, 141] and cuprous iodide [142] had anti-inflammatory activity and that Cu(II)(salicylate)$_2$ had fever-lowering effects [81, 82] in various models of inflammation and fever. In 1969, Bonta [143] and Laroche [144] reported that cupric carbonate ($Cu(OH)_2CuCO_3$) and cupric complexes of acetic, lauric, oleic, caprylic, butyric, sebasic, lipoic and cinnamic acids [144] were also effective in animal models of inflammation.

In 1974 [92] and again in 1976 [22, 145], it was suggested that copper complexes of clinically used anti-arthritic drugs were formed *in vivo* and that these complexes were responsible for the beneficial anti-arthritic effects of these drugs. This suggestion was supported by observations that copper complexes of many non-anti-inflammatory complexing agents, including amino acids, heterocylic carboxylic acids, amines, and other classes of chemical compounds, had anti-inflammatory activity in recognized animal models of inflammation: carrageenan, paw oedema, cotton wad granuloma, and rat polyarthritis. In addition, copper complexes of antiarthritic drugs, including salicylic acid, acetylsalicylic acid, 1-phenyl-5-aminotetrazole, 2-[3-trifluoromethylphenyl]aminonicotinic acid, penicillamine and several corticoids were found to be more active than their parent drugs. All of these copper complexes were found to be more active than either inorganic copper salts or their parent complexing agents, regardless of whether or not the parent complexing agent had anti-inflammatory activity.

Data demonstrating these remarkable observations are presented in

Table 6.3. COMPARISON OF ANTI-INFLAMMATORY ACTIVITIES OF COPPER COMPLEXES WITH THEIR PARENT LIGAND AND COPPER ACETATE[a] [22]

Compound	*Carrageenan paw oedema*	*Cotton wad granuloma*	*Polyarthritis*	*Copper (%)*
$Cu(II)_2(acetate)_4(H_2O)_2$	A[b] at 0.02	I[c] at 0.30	I at 0.089	31.8
Anthranilic acid	I at 1.46	n.t.[d]	I at 0.219	
3,5-DIPS acid	I at 0.90	n.t.	I at 0.135	
Cu(II)(anthranilate)$_2$	A at 0.02	A at 0.07	A at 0.003	18.9
Cu(II)(3,5-DIPS)$_2^{[e]}$	A at 0.02	A at 0.10	A at 0.002	12.5
Aspirin	A at 0.36	A at 1.11 (i.g.)	A at 0.033	
$Cu(II)_2(aspirinate)_4$	A at 0.01	A at 0.01	A at 0.001	15.0
D-Penicillamine	I at 1.34	I at 0.67	I at 0.201	
Cu(I)D-pen$(H_2O)1.5$	A at 0.03	A at 0.04	n.t.	26.7
Cu(II)(D-pen disulphide)$(H_2O)_2$	A at 0.01	A at 0.03	A at 0.040[f]	15.4

[a] All compounds were given by subcutaneous injection unless indicated as intragastric (i.g.) and expressed as millimole per kilogram of body weight (mmol/kg).

[b] Lowest active dose tested.

[c] Inactive at dose tested.

[d] Not tested.

[e] This complex has recently been shown to be a binuclear complex, $Cu(II)_2(3,5\text{-DIPS})_4$ (see p. 546).

[f] Only dose of this penicillamine complex tested.

Table 6.3. $Cu(II)_2(acetate)_4$ was found to be active in the initial test (carrageenan paw oedema) for anti-inflammatory activity, but inactive in the two follow-up anti-inflammatory screens (cotton wad granuloma and polyarthritis). Cupric chloride had no activity in any of these models of inflammation. Ligands such as anthranilic acid and 3,5-diisopropylsalicylic acid (3,5-DIPS) which were anticipated to be inactive were found to be so. However, their copper complexes were found to be potent anti-inflammatory agents in all three models of inflammation. These observations supported the notion that complexed copper is a more active anti-inflammatory form of copper and led to the suggestion that copper complexes of active anti-inflammatory agents might be more active than their parent anti-inflammatory drugs.

Representative data from the original report comparing aspirin and D-penicillamine with their copper complexes are also presented in *Table 6.3.* The original presentation of these results was based upon weight of compound/kilogram of body weight required to produce a significant reduction in inflammation [22]. Comparison of aspirin with $Cu(II)_2(aspirinate)_4$ on this basis showed that $Cu(II)_2(aspirinate)_4$ was 8-times as effective as aspirin in the carrageenan paw oedema model of inflammation and greater than 5-times as

effective as aspirin in the polyarthritis model of inflammation. However, as shown in *Table 6.3*, comparison of these two compounds based upon number of molecules required to produce these anti-inflammatory effects reveals that $Cu(II)_2(aspirinate)_4$ is more than 30-times as effective as aspirin in these two models of inflammation. Since D-penicillamine is ineffective in all three models of inflammation, the marked increase in activity for its copper complexes is clear, based upon weight or number of molecule comparisons.

These data show that these complexes are much more effective than their parent drugs and support the hypothesis that active metabolites of anti-arthritic drugs are their copper complexes. Since amounts of copper in these complexes do not appear to correlate with activity, it is suggested that pharmacologic activity may be better correlated with their physicochemical properties.

Acute toxicity studies of copper complexes were carried out early in the course of this work [22]. These demonstrated that anti-inflammatory copper complexes were less toxic than inorganic forms of copper as well as their parent anti-arthritic drugs. The oral LD_{50} values for copper aspirinate were found to be 1.06 ± 0.26 and 1.16 ± 0.33 mmol/kg, respectively, for male and female Sprague-Dawley rats. Chronic toxicity studies using 0.12 mmol (100 mg)/kg given orally to male Sprague-Dawley rats 5 days per week for 3 months did not affect growth, survival, plasma copper or zinc concentrations, or copper and zinc concentrations in 15 tissues, including skin and fur [146]. Histopathological examination of all tissues, except skin and fur, at the light microscopic level revealed no evidence of pathological changes except for an increase in Kupffer cell number in the liver of those animals that were killed at the end of the 3 month treatment period. Kupffer cell number progressively decreased in livers of animals killed at the end of the subsequent 2 months of this 5 month study. The projected human dose of copper aspirinate is 3–6 μmol/kg daily with a decrease to some maintenance dose with remission. This regimen seems to be safe enough to correct what may be, in part, a copper deficiency or inadequacy disease, since the recommended safe daily intake of copper is 47 μmol/day.

The observation that copper complexes have anti-inflammatory activity has been confirmed and extended by many others with reports that the same and additional copper complexes listed in *Table 6.4* have anti-inflammatory activity in an even greater variety of animal models of inflammation: carrageenan, kaolin, dextran, hydroxyapatite and concanavolin A (Con A) paw oedemas, cotton wad granuloma, polyarthritis and ultraviolet erythema. Data presented for copper complexes and their parent ligands in all subsequent reports when compared on a molar basis also show marked increases in anti-inflammatory

Table 6.4. COPPER COMPLEXES STUDIED AS ANTI-INFLAMMATORY AGENTS

Compound	*Reference*
Inorganic copper compounds	
Metallic Cu	200
Cu(I)I	142
$Cu(II)Cl_2$	22,154,155,164
Cu(II)O	154–155
$Cu(II)(OH)_2CuCO_3$	143,145
Cu(I)Cl	164
$Cu(I)_2O$	164
$Cu(II)(pyridine)_2(Cl)_2$	22,164
$Cu(II)(morpholine)_2(HCl)_2$	22
$Na_2Cu_2(S_2O_2)_2$	150
$Cu(I)(thioacetamide)_4Cl$	150
$Cu(I)(MeCN)_4(CO)_4$	150
Cu(I)(Cl)(dimethylsulphoxide)	150
$Cu(II)(thiourea)_2SO_4$	164
Aliphatic carboxylic acids	
$Cu(II)_2(acetate)_4$	22,144,153–155,164,164a
$Cu(II)_2(acetate)_4(pyridine)_2$	164
Cu(II)-(citrate)	149
Cu(II)-(butyrate)	144,183
Cu(II)-(cinnamate)	144
Cu(II)-(caprylate)	144
Cu(II)-(lactate)	183
Cu(II)-(laurate)	144
Cu(II)-(lipoate)	144
Cu(II)-(oleate)	144
Cu(II)-(sebasate)	144
Cu(II)-glycyrrhizic acid	158
Amino acids	
Cu(II)(L-tryptophanate)$_2$	22,179,180,192
Cu(II)(D-tryptophanate)$_2$	22,179,180
$Cu(I)_n$(D-penicillamine)$_n$	22,150,164,198
$Cu(II)_2$(D-penicillamine disulphide)$_2$	22,150
Cu-D-penicillamine	147,148,157
$Na_5Cu(I)_8Cu(II)_6$(D-penicillamine)$_{12}Cl$	150
Cu(I)-(*N*-acetylpenicillamine)	183
Cu(II)(D-aspartate)	22
Cu(II)(L-aspartate)	22
Cu(II)(L-lysinate)$(Cl)_2$	22
Cu(II)(glycinate)$_2$	149,150,198
$Cu(II)(L\text{-histidine})_2NO_3$	164
Cu(II)(D- and L-histidinate)$_2$	179,180
Cu(II)(D- and L-cystinate)	179,180,198
Cu(II)(alaninate)$_2$	188

Table 6.4. continued

Compound	*Reference*
Cu(II)(phenylalaninate)$_2$	192
Cu(II)-(glutamate)	192
Anthranilic acids	
Cu(II)(anthranilate)$_2$	22,164,170
Cu(II)$_2$[2-[3-(trifluoromethyl)phenyl]aminonicotinate]$_4$	22,153,163,183
Cu(II)$_2$(*N*-2,3-dimethylphenylanthranilate)$_4$	156
Cu(II)$_2$(*N*-3,4-dimethylphenylanthranilate)$_4$	193
Cu(II)-(*N*-2-chloro-4-pyrimidinoanthranilate)	156
Cu(II)(*N*-salicylideneanthranilate)$_2$	191
Cu(II)-(*N*-phenylanthranilate)	183
Aryl acetic acids	
Cu(II)$_2$[1-(4-chlorophenyl)-2-,5-dimethyl-1*H*-pyrrole-3-acetate]$_4$	153
Cu(II)$_2$[1-(p-chlorobenzoyl)-5-methoxy-2-methyl-3-indolylacetate]$_4$	153,164a,181,194
Cu(II)$_2$(4-cyclopropylmethyleneoxy-3-chlorophenylacetate)$_4$	162
Cu(II)$_2$(3-*p*-chlorophenyl)-1-phenylpyrrole-4-acetate)$_4$	194
Aryl propionic acids	
Cu(II)$_2$[2-(3-benzoylphenyl)propionate]$_4$	153
Cu(II)$_2$[(+)-2-(6-methoxy-2-naphthyl)propionate]$_4$	153,187
Cu(II)-2-[2-(6-methoxynaphthalene)]propionate	159
Cu(II)-[2-(4-isobutylphenyl)propionate]	160,161,201
Cu(II)-[2-(3-benzophenone)propionate]	162
Benzoic acids	
Cu(II)-(benzoate)	183
Cu(II)-(3-hydroxybenzoate)	183
Cu(II)-(4-hydroxybenzoate)	183
Cu(II)-2-chloro-4-(*o*-carboxyphenylamino)pyrimidine	156
Cu(II)(2-selenobenzoate)$_2$	167
Cu(II)-2,4-di(*p*-carboxyphenylamino)pyrimidine	156
Cu(II)-2,4-di(*m*-carboxyphenylamino)pyrimidine	156
Cu(II)-2,4-di(*o*-carboxyphenylamino)pyrimidine	156
Cu(II)$_2$[2-(2-carboxyphenyl)amino-4-(2-carboxyphenyl)aminopyrimidine]$_2$	169
Cu(II)$_2$[2-carboxyphenyl)amino-4-(2,3-dicarboxyphenyl)aminopyrimidine]	169

(*continued*)

Table 6.4. continued

Compound	*Reference*
$Cu(II)_2$[2-(2-carboxyphenyl)amino-4-(2,4-dicarboxyphenyl)aminopyrimidine]	169
$Cu(II)_2$(2-thienylglyoxylate)$_4$	191
$Cu(II)_2$(2-methoxybenzoate)$_4$	181
$Cu(II)_2$(4-methoxybenzoate)$_4$	181
$Cu(II)_2$(2-furanoate)$_4$	181
Cu(I)-(2-mercaptobenzoate)	183
Corticoids	
$Cu(II)_3$(hydrocortisone-21-phosphate)$_2$	22
$Cu(II)_2$(hydrocortisone-21-hemisuccinate)$_4$	22
$Cu(II)_3$(dexamethasone-21-phosphate)$_2$	22
Ethylenediamines	
Cu(II)(H_2-ethylenediaminetetraacetate)	163
K_2Cu(II)(ethylenediaminetetraacetate)	163
Cu(II)(ethylenediamine)$_2(Cl)_2$	163,189
Cu(II)(ethylenediamine)$_2(NO_3)_2$	163
Cu(II)(ethylenediamine)$_2SO_4$	163
Cu(II)-(ethylenediamine)-(malonate)	183
Cu(II)-(ethylenediamine)-(thiocyanate)$_2$	183
Cu(II)-(1,2-glycylethane)	192
Cu(II)-(1,2-valylethane)	192
Cu(II)-1,2-leucylethane)	192
Cu(II)-(1,2-tryptophanylethane)	192
Heterocyclic carboxylic acids	
Cu(II)(1-carboxyisoquinoline)$_2$	22
$Cu(II)_2$(2-carboxyindole)$_3$(acetate)	22
Salicylates	
Cu(II)(3,5-diisopropylsalicylate)$_2$	22,164a
$Cu(II)_2$(acetylsalicylate)$_4$	22,151–155,163,164a,188,189
Cu(II)(salicylate)$_2$	145,152,170,188,194
Cu(II)(salicylate)$_2$(ethanol-glyceral) or (dimethylsulphoxide)	165–170,183
Cu(II)(pyridine)$_2$(acetylsalicylate)$_2$	163
Cu(II)-(4-methylsalicylate)	183
Cu(II)-(5-methylsalicylate)	183
Cu(II)-(4,5-dimethylsalicylate)	183
Cu(II)-(*O*-salicylsalicylate)	183
Cu(II)-(ethylsalicylate)	183
Cu(II)-(4-acetamidosalicylate)	183
Cu(II)-(salicylurate)	183

Table 6.4. continued

Compound	*Reference*
Cu(II)-(diflunisal)	183
Cu(II)-(1-hydroxy-2-naphthoate)	183
Cu(II)-(salicylanilide)	183
Cu(II)-(salicylaldehyde)	183
Cu(II)-(salicylaldoxime)	183
Cu(II)-(salicylhydroxamate)	183
Sulfonamides	
Cu(II)(*N*-[[(2-phenyl-2-hydroxyethyl)amino]ethyl]-*p*-toluenesulphonamide)$_2$	163
Cu(II)(*N*-[[(1-phenyl-2-hydroxyethyl)amino]ethyl]-*p*-toluenesulphonamide)$_2$	163
Cu(II)(*N*-[1-phenyl-2-(2-iminothiazolidin-3-yl)ethyl]-*p*-toluenesulphonamide)$_2$	163
Tetrazoles	
Cu(II)$_2$(1-phenyl-5-aminotetrazole)$_2$(acetate)$_4$	22
Cu(II)$_2$(1-phenyl-5-aminotetrazole)$_4$(Cl)$_2$	22
Cu(II)-(1-phenyl-5-aminotetrazole)	183
Thiols	
Na_2Cu(I)thiomalate	150
Cu(I)dithiodiglycol	150
NaCu(I)(3-*N*-allythiouredobenzoate)	140,141
Cu(I)-(2-mercaptoethanol)	183
Triphenylphosphines	
Cu(I)(triphenylphosphine)$_3$Cl	163
Cu(I)(triphenylphosphine)$_2$(diethyldithiocarbamate)	163
Cu(I)(triphenylphosphine)(acetylsalicylate)	164
Miscellaneous	
Cu-ascorbate	150
Cu(II)(aminophenazol)$_2$(ClO_4)	199
Cu(II)(antipyrene)(ClO_4)	181,199
NaCu(II)-(chlorophylline)	185
Cu(II)-(3,4-dimethoxycinnamylhydroxamate)	189
Cu(II)(*N*-2-amino-2-deoxy-α,β-D-glucopyranose salicylaldimine)	191
Cu(II)(azopropazone)$_2$	196
Cu(II)(D-mandelate)$_2$	191
Cu(II)(L-mandelate)$_2$	191
Cu(II)(D,L-mandelate)$_2$	191

(*continued*)

Table 6.4. continued

Compound	*Reference*
Cu(II)[(−)-2,3,5,6-tetrahydro-6-phenylimidazo(2,1-*b*)thiazole]$_2$Cl$_2$	157,164
Cu(II)(diethyldithiocarbamate)	164
Cu(II)(2-amino-2-thiazoline)$_4$Cl$_2$	164
Cu(II)[4-hydroxy-3-(5-methyl-3-isoxazolocarbamyl-2-methyl-2*H*-1,2-benzothiazine-1,1-dioxide]$_2$	195
Cu(II)((5-dimethylamino)-9-methyl-2-propyl-1*H*-pyrazolo[1,2-*a*][1,2,4]benzotriazine-1,3(2*H*)-dione)$_2$	196
Cu(II)-α-tropolone)	183
Cu(II)-(2-hydroxyacetophenone)	183
Cu(II)-(trifluoroacetylacetone)	183
Cu(II)-(hexafluoroacetylacetone)	183
Cu(II)-(β-oxo-2-thiophenepropionitrile)	175
Cu(II)-(phenylbutazone)	173,174,183

activity for copper complexes. A comparison of copper, gold, and silver thiomalate and thiosulphate complexes in these models of inflammation revealed that the copper complexes were effective while the gold and silver complexes were virtually inactive.

All of the anti-inflammatory activities reported for compounds listed in *Table 6.4* were obtained following oral or parenteral administration. Even mixtures of copper salts and ligands or copper complexes in aqueous ethanol or glycerol-dimethylsulphoxide solutions have recently been shown to have anti-inflammatory activity in a variety of animal models of inflammation following topical application [165–170, 183]. These formulations were developed following the demonstration that copper would be absorbed through the skin [171, 172]. Following topical application, ^{64}Cu was measured in the skin and faecal excretion of ^{64}Cu was measured following treatment with ^{64}Cu(II)(salicylate)$_2$ and ^{64}Cu(II)(phenylbutazone) [173, 174]. Unfortunately, mixtures of inorganic copper salts and ligands seem to be always less effective than administration of the preformed complex.

A number of new copper complexes have also been found to have anti-inflammatory activity. Copper(II)(β-oxo-2-thiophenepropionitrile) is effective in treating rat polyarthritis [175]. The copper complex of glycyl-L-histidyl-L-lysine, Cu(II)(GHL), is claimed to have anti-inflammatory activity and increase the rate of wound healing [176, 177] consistent with earlier reports of increased gastric wound healing rates associated with Cu(II)(aspirinate)$_4$ and Cu(II)(tryptophanate)$_2$ treatment [178]. Copper(II)(L-histidinate)$_2$, Cu(II)-

(D-histidinate)$_2$ inhibited dextran- and Con-A-induced anaphylactoid reactions in rats, while their parent ligands were ineffective [179]. Copper(II)(L-histidinate)$_2$ also inhibited dextran and carrageenan paw oedemas, ultraviolet erythema and established rat polyarthritic inflammations without causing injection-site irritation [180]. Dextran-induced release of histamine from mast cells *in vitro* and *in vivo* was also significantly prevented by Cu(II)(L-histidinate)$_2$, perhaps by stabilizing both mast cells and granule membranes, which was suggested to possibly stabilize lysosomal membranes and prevent release of hydrolytic enzymes [179]. Co-administration of this copper complex with histamine or 5-hydroxytryptamine did not inhibit histamine- or 5 HT-induced responses, suggesting no mediator blockade effects but prevention of mediator release. Copper(II)(L- or D-histidinate)$_2$ inhibited Con-A-induced paw oedema in rats at a dose of 2 mg/rat (ID_{50}, $P < 0.01$). On the other hand, histidine potentiated Con-A-induced and dextran-induced increases in paw volume [179, 180] and had no effect on carrageenan paw oedema [180]. Copper(II)(D- or L-histidinate)$_2$ also inhibited kaolin paw oedema, which is considered to be mediated by bradykinin and prostaglandin releases, while histidine had no effect. Copper(II)(L-tryptophanate)$_2$ inhibited both carrageenan- and Con-A-induced oedemas, but stimulated dextran-induced oedema [180], perhaps due to its lack of solubility. Copper(II)(cystinate) inhibited dextran-, carrageenan- and Con-A-induced oedemas and it was much more effective than cysteine alone, on a molar basis [180]. Copper(II)(D- or L-cystinate) and Cu(II)(D- or L-histidinate)$_2$ inhibited all three models of inflammation. Cu(II)(D- or L-tryptophanate)$_2$ inhibited carrageenan- and Con-A-induced inflammations but potentiated the dextran-induced inflammation. This potentiation may have been due to a facilitation of the inflammation associated with dextran [180].

Copper(II)$_2$(2-methoxybenzoate)$_4$, Cu(II)$_2$(4-methoxybenzoate)$_4$, Cu(II)$_2$-(2-furanoate)$_4$, and Cu(II)-(antipyrine) at doses of 10 mg/kg (i.p.) were more effective than antipyrine in inhibiting dextran paw oedema and less acutely toxic than antipyrine [181, 182].

In a large study, many ligands were mixed with $Cu(OH)_2$ or Cu_2(acetate)$_4$ in glycerol-ethanol or dimethylsulphoxide and studied in the carrageenan, hydroxyapatite and polyarthritis models of inflammation to determine anti-inflammatory activity following topical application [183]. Many of these mixtures were found to have anti-inflammatory activity and comparisons of Cu(II)(salicylate)$_2$, Cu(II)$_2$(niflumate)$_4$ and Cu(II)(phenylbutazone)$_2$ with their parent ligand in the polyarthritis model revealed that these complexes were more effective than their parent ligands, which were inactive or much

less active, following topical application. Mixtures of thiols or ascorbic acid plus betaine or sulphobetaine with a cuprous halide in alcohols were also found to be topically effective antifungal, antiviral and anti-inflammatory mixtures [184]. Sodium copper(II) chlorophylline mixed with glycyrrhetinic acid was suggested to be useful in treating buccal cavity inflammation [185].

Induction of carrageenan rat paw oedema was found to decrease liver and brain copper contents and increase plasma and paw (inflammation site) copper levels [186]. In this study, aspirin inhibited oedema faster than a mixture of copper and aspirin, which is consistent with unpublished observations that such mixtures are always less effective than $Cu(II)_2(aspirinate)_4$. However, the rate of oedema inhibition was faster with a mixture of copper and naproxen than with the sodium salt of naproxen. Serum copper decreased after administration of anti-inflammatory drugs, which may bear on the suggestion that these drugs do form complexes with copper *in vivo*. Induction of gastric bleeding was greater for a mixture of copper and salicylic acid compared with sodium salicylate, whereas bleeding was greater for sodium naproxen than for the mixture of copper and naproxen. These observations may be consistent with the lack of complexation in the mixture of copper and sodium acetylsalicylate so that there was less anti-inflammatory activity and more gastric irritation, as copper complexes have been found to be more effective as anti-inflammatory agents than their parent ligands and these complexes are not gastric irritants but have antiulcer activity (see Section on gastrointestinal ulcers). Mixtures of this sort have been found to be irritating to the gastric mucosae [152, 187]. The mixture of copper and naproxen may have produced sufficient complex so as to prevent the observation of gastric irritation. Since mixtures of copper and anti-inflammatory drugs always leave open the question as to whether or not complexation occurs before treatment, such mixtures should never be used to evaluate the hypothesis that copper complexes have anti-inflammatory activity, lack gastric irritant activity and have antiulcer activity.

Lewis, Smith and Brown compared the oral anti-inflammatory activities of $Cu(II)(ethylenediamine)(Cl)_2$, $Cu(II)(L\text{-alaninate})_2$, and $Cu(II)_2(aspirinate)_4$ in the kaolin paw oedema model of inflammation [188] in adressing the question as to whether or not mixtures of inorganic copper salts and ligands provide the form of copper needed for maximal anti-inflammatory activity and/or are more gastric or parenterally irritating than synthesized complexes. Upon examination of the counter-irritant or gastric irritation mechanism of action hypotheses, they found a lack of correlation between anti-inflammatory activity and local irritation, suggesting a more direct mechanism of anti-inflammatory action. A more detailed analysis of forms of copper, the exact

function of copper-bonding species, and its interaction with other trace elements were suggested as needed to further understand the importance of these changes. More detailed examination of differential effects of anti-inflammatory drugs on elevated levels of plasma copper was also suggested to possibly provide valuable information [188].

Copper(II)(3,4-dimethoxycinnamylhydroxamate) was found to be less effective against carrageenan paw oedema than its parent ligand, which was suggested to be due to the polymeric nature of the complex and the attendant lack of solubility [189]. This problem may have been overcome with a better formulation using a non-ionic suspending agent so that absorption might have been facilitated following administration.

Studies of Cu(II)(*N*-2-amino-2-deoxy-α,β-D-glucopyranose salicylaldimine) using doses of 10 to 30 mg/kg (s.c.) showed 25–53% reduction of the cotton wad granuloma and an increase in serum copper soon after treatment [190]. Copper(II)$_2$(mandelate)$_4$, Cu(II)$_2$(thienylglyoxylate)$_4$, and Cu(II)$_2$(*N*-salicylylanthranilate)$_4$ were also found to be effective in this model of inflammation in doses ranging from 10–200 mg/kg (s.c.) when given for 7 days [191]. It was suggested that these ligands modulated the pharmacologic effect of copper.

Katz reported that solutions of a group of ethylene bridged amino acids mixed with $CuCl_2$ and pH adjusted to 7.6 with sodium carbonate were effective in inhibiting carrageenan paw oedema, while mixtures containing $MnCl_2$ instead of $CuCl_2$ were ineffective [192]. Both mixtures had SOD-mimetic activity, but mixtures containing copper were effective in disproportionating superoxide at a 10-fold lower concentration than those mixtures containing $MnCl_2$. The notion that these mixtures contained the same structural species as obtained via synthesis and characterization of 1:1 copper complexes [134] was not substantiated with evidence for this effect, and partition coefficient determinations revealed greater hydrophilicity for the copper-containing components rather than greater lipophilicity as expected for non-charged complexes. Consequently, the observed anti-inflammatory activities may be only a fraction of the activity observed following administration of the synthesized 1:1 complexes.

The list of compounds presented in *Table 6.4* includes copper complexes of well-known antiarthritic drugs, including salicylic acid, aspirin, diflunisal, niflumic acid, D-penicillamine, hydrocortisone, dexamethasone, dimethyl-sulphoxide, clopirac, ketoprofen, ibuprofen, (+)-naproxen, indomethacin, mefenamic acid, thiomalic acid, phenylbutazone, lonazolac, isoxicam and azopropazone. While there has been much discussion concerning the qualitative and quantitative activities of these complexes [136, 152–156, 164, 187], it is

true that copper complexes of nonanti-inflammatory complexing agents do have anti-inflammatory activity and, where data have been provided, copper complexes of anti-arthritic drugs have been found to be more active or more effective anti-inflammatory agents than their parent drugs. It is always found that, when activities of parent ligands are compared with activity of their complexes on a molar basis, these complexes are active at a dose lower than that of the parent ligand. This is in part supported by observations that many of these and other copper complexes have been studied and found to have antiulcer activity while the parent drugs are ulcerogenic (see next section, on gastrointestinal ulcers).

ANTIPYRETIC ACTIVITIES OF COPPER COMPLEXES

In the early and mid 1960's, Schubert reported that Cu(II)(salicylate)$_2$ was more effective than either Cu(II)Cl$_2$ or salicylic acid in lowering yeast-induced fever in rats [81, 82]. Hac and Gagalo [192a] recently reported that Cu(II)(salicylate)$_2$ is much more antipyrogenic than sodium salicylate in a rabbit model of endotoxin-induced fever produced by the injection of a lipopolysaccharide pyrogen derived from *Escherichia coli*. Copper(II)(salicylate)$_2$ reduced body temperature at various ambient temperatures ranging from 5 to 28 °C, while sodium salicylate did not, and a central neuro-effector pathway effect was invoked to account for these results and the observation that Cu(II)(salicylate)$_2$ reduced body temperature in afebrile rabbits. Pifferi reported that Cu(II)$_2$(4-cyclopropylmethyleneoxy-3- chlorophenylacetate)$_4$ was four times as effective, on a molar basis, as an antipyretic agent as its parent ligand in bactopeptone-injected rats [162]. Cu(II)$_2$(2-methoxybenzoate)$_4$, Cu(II)$_2$-(2-furanoate)$_4$, and Cu(II)-(antipyrine) were also found to have greater antipyretic activity than either antipyrine or phenylbutazone in endotoxin-treated rabbits [181, 182]. Prolonged antipyretic activity has also been reported for Cu(II)$_2$(*N*-3,4-dimethylphenylanthranilate)$_4$ [193].

ANALGESIC ACTIVITIES OF COPPER COMPLEXES

Observations by Pifferi [162] that Cu(II)$_2$(4-cyclopropylmethyleneoxy-3-chlorophenylacetate)$_4$ had nearly 10-times the analgesic activity of its parent ligand in a yeast-induced paw oedema model of pain (ED_{50} value 0.018 m mol/kg i.p. and i.g.) and the phenylquinone writhing pain model (ED_{50} value 0.023 m mol/kg i.g.) and that the copper complex was more effective than phenylbutazone in both pain models was missed in earlier reviews [41, 84]. A subsequent comparison of Cu(II)-(3,4-dimethoxycinnamylhydroxamate)

with its parent ligand showed tnat the complex was only half as effective as its ligand in the phenylquinone writhing pain model which was attributed to the polymeric nature of this complex [189].

More recent studies have clearly shown that Cu(II)(anthranilate)$_2$, Cu(II)(salicylate)$_2$, Cu(II)$_2$(3,5-DIPS)$_4$, Cu(II)$_2$(aspirinate)$_4$, Cu(II)$_2$(niflumate)$_4$, and Cu(II)$_2$(indomethacin)$_4$ are more effective analgesics than their parent ligands [93]. As shown in *Table 6.5*, copper complexes of salicylic acid, 3,5-DIPS, aspirin, niflumic acid and indomethacin were more effective than their parent compounds and $CuCl_2$ or Cu$_2$(acetate)$_4$ in writhing and polyarthritic pain models. In addition, the copper complex of indomethacin was found to be as effective as morphine in both pain models and the amount of copper

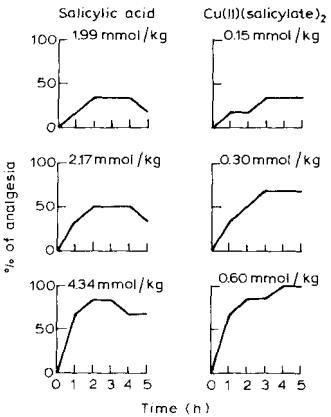


Figure 6.4. Time-course of analgesia due to salicylic acid or Cu(II)(salicylate)$_2$ in the polyarthritic pain model [93].

in the 1 μmol dose of Cu(II)$_2$(indomethacin)$_4$ is only one-fifth the recommended safe daily intake of copper.

To evaluate the time-course of analgesia associated with a non-steroidal anti-inflammatory agent and its copper complex, salicylic acid and its complex were compared in the polyarthritis pain model following oral administration in 5% gum arabic. Data presented in *Figure 6.4* show that analgesic activity increased in a dose-related manner and that Cu(II))(salicylate)$_2$ was 7- to 10-times as effective as salicylic acid [93]. In addition, the analgesic activity of Cu(II)(salicylate)$_2$ appears to be more sustained than the analgesic activity of salicylic acid, since analgesia associated with salicylic acid treatment began to decline in the 3–4 h interval following treatment, while Cu(II)(salicylate)$_2$analgesia was maintained or increased throughout the entire 5 h post-

Table 6.5. ANALGESIC EFFECTS OF PARENT LIGANDS AND COPPER COMPLEXES ON ACETIC ACID-INDUCED WRITHING PAIN IN MICE AND POLYARTHRITIC PAIN IN RATS [93]

Compound	*Writhing pain*			*Polyarthritic pain*		
	Doses[a] (mmol/kg)	*% Inhib.*	*ED_{50} value (mmol/kg) (95% C.L.)*	*Doses[a] (mmol/kg)*	*% Inhib.*	*ED_{50} value (mmol/kg) (95% C.L.)*
Salicylic acid	2.17	38*	>2.17	0.72	33	1.83
				1.44	43	(0.49 –6.81)
				2.88	60	
Cu(II)(salicylate)$_2$	0.30	20	1.53	0.15	30	0.25
	0.59	27	(0.61–3.80)	0.30	47	(0.13–0.48)
	1.18	46*		0.59	90	
3,5-DIPS	0.22	19	0.4	0.45	27	>0.90
	0.45	56**	(0.29–0.67)	0.90	43	
	0.90	78***				
Cu(II)(3,5-DIPS)$_2$	0.20	26	0.43	0.40	40	>0.40
	0.40	56*	(0.20–0.91)			
	0.79	62***				
Aspirin	0.21	32	0.48	0.56	30	1.41
	0.42	41*	(0.22–1.04)	1.11	40	(0.50–3.96)
	0.84	67***		2.22	63	

$Cu(II)_2$(aspirinate)$_4$	0.09	32*	0.14	0.03	13	0.09
	0.18	59**	(0.09–0.25)	0.06	30	(0.04–0.21)
	0.36	80***		0.12	60	
Niflumic acid	0.13	38*	0.21	0.18	23	0.48
	0.27	51**	(0.11–0.40)	0.35	43	(0.19–1.20)
	0.53	80***		0.71	60	
$Cu(II)_2$(niflumate)$_4$	0.06	33*	0.10	0.16	37	>0.16
	0.12	57***	(0.05–0.20)			
	0.24	73***				
Indomethacin	0.007	27	0.01	0.007	23	0.02
	0.014	60**	(0.01–0.03)	0.014	43	(0.01–0.03)
	0.028	68***		0.028	70	
$Cu(II)_2$(indomethacin)$_4$	0.001	38	0.002	0.001	20	0.002
	0.002	53**	(0.001–0.003)	0.002	40	(0.001–0.003)
	0.004	75***		0.003	87	
$CuCl_2$				2.24	0	>2.24
$Cu(II)_2$(acetate)$_4$				0.83	0	>0.83
Morphine hydrochloride[b]	0.001	35	0.002	0.001	20	0.002
	0.002	44*	(0.001–0.003)	0.002	40	(0.001–0.005)
	0.004	89***		0.004	60	

[a] Administered orally in 5% propylene glycol and 1.4% poly(vinyl alcohol) in water.
[b] Administered subcutaneously in saline.
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus vehicle treated group.

treatment interval. This sustained analgesic effect may be the result of slower and prolonged gastric absorption of Cu(II)(salicylate)$_2$ in comparison with salicylic acid, due to its greater lipophilicity and slow release from the 5% gum arabic vehicle.

As shown in *Table 6.6*, copper complexes of amino acids were also effective analgesics, while their ligands were ineffective in the acetic acid-induced pain

Table 6.6. ANALGESIC EFFECTS OF AMINO ACIDS AND THEIR COPPER COMPLEXES ON ACETIC ACID INDUCED WRITHING IN MICE AND POLYARTHRITIC PAIN IN RATS [93]

Compound	*Oral (mmol/kg)*[a]	*% Inhibition*	*ED_{50} (mmol/kg) (95% C.L.)*
L-Alanine	4.49	13.5	>4.49
Cu(II)(L-alaninate)$_2$	0.42	22.3	0.92
	0.83	47.9**	(0.54–1.56)
	1.67	70.7**	
	0.42[b]	0	>0.42
L-Cysteine	3.30	−4.8	>3.30
Cu(II)(L-cystinate)	0.33	34.9*	>0.33
	0.33[b]	0.0	>0.33
Glycine	5.33	35.6*	>5.33
Cu(II)(glycinate)$_2$	1.89	48.4**	>1.89

[a] Administered in 5% gum arabic in saline.
[b] Activity in adjuvant-induced arthritic pain.
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus vehicle-treated group.

model but ineffective in the adjuvant-induced arthritic pain model. Even though Cu(II)(L-alaninate)$_2$, Cu(II)(L-cystinate) and Cu(II)(glycinate)$_2$ were less active than the other copper complexes, including Cu(II)Cl$_2$ and Cu(II)$_2$(acetate)$_4$, the fact that each of these complexes was more active than its parent ligand suggests that this class of copper complexes may also be important physiological modulators of nociception.

Dissociation of these copper complexes in the stomach cannot be used to account for analgesic activities of these complexes, as their ligands and more freely dissociable forms of copper, CuCl$_2$ and Cu$_2$(acetate)$_4$, have less activity than is observed for these complexes. In addition, copper complexes have antisecretory activity and decrease gastric acidity [22, 41, 84], which would impede dissociation.

It is phenomenal that the copper complex of acetic acid is effective in preventing pain in the writhing model, since the injection of acetic acid, its

ligand, is used to produce pain in this model. In addition, irritant-induced anti-inflammatory and concomitant stress-induced analgesic activity cannot be used to explain these results, since the most likely irritant, $CuCl_2$, was the least effective compound.

These data are consistent with the notion that non-steroidal anti-inflammatory agents form copper complexes *in vivo* [22] and support the possibility that copper complex formation *in vivo* accounts for the analgesic activity of these anti-inflammatory agents as well. The observation that copper complexes are absorbed and mediate analgesia following tissue distribution suggests physiological and biochemical roles for copper in modulating nociception.

Dopamine-β-hydroxylase (DβH) inhibitors such as diethyldithiocarbamate (DDC) and 1-phenyl-3-(2-thiazoyl)-2-thiourea (PTT) are known to remove copper from this copper-dependent enzyme and, in addition, potentiate antagonist-reversible morphine analgesia [202, 203]. Watanabe, Matsui and Iwata [202] were the first to report that DDC elevated pain threshold and potentiated morphine analgesia in the tail-clip and hot-plate pain models. Bhargava and Way [203] subsequently reported that PTT also elevated pain threshold and reversed or prevented morphine tolerance by potentiating analgesia in naïve and morphine-tolerant mice and prevented symptoms of abrupt morphine withdrawal and symptoms of naloxone-precipitated withdrawal.

Both DβH inhibitors produced dose-related increases in CNS copper content [203–205]. With DDC treatment, whole brain copper content was significantly elevated (160% of normal) while serum copper and ceruloplasmin levels were decreased to 85% of normal. Ceruloplasmin contains 6 atoms of copper and it is the principal copper containing component of blood plasma or serum. Marked increases in copper were found in the cerebral cortex (136%), brain stem (147%), and spinal cord (162%) with only a slight increase in the cerebellum (12%). Accumulation in the CNS was associated with morphine potentiation by DDC and morphine analgesia. However, algesic states produced by levallorphan, morphine plus levallorphan, and morphine tolerance caused decreases in all tissues of the brain [204, 205]. Lowered copper content of the cerebral cortex in morphine-tolerant rats and their decreased pain threshold [206] is consistent with severe pain produced in rats made copper-deficient by treating with penicillamine [207]. Pretreatment of normal rats with penicillamine is also known to inhibit morphine and β-endorphin analgesia. These observations support the suggestion that a lipophilic copper-DDC complex, formed *in vivo*, penetrates the blood-brain barrier and mediates analgesia. This suggestion has recently been affirmed with *in vitro* studies showing that DDC added to blood, serum or plasma immediately forms $Cu(II)(DDC)_2$ with copper from serum or plasma proteins [208]. The

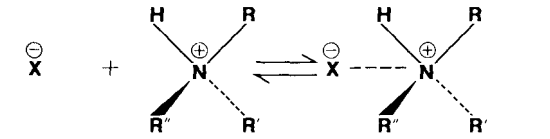
report that DDC decreases ceruloplasmin activity by removing copper provides additional support for the formation of $Cu(II)(DDC)_2$ *in vivo* [204].

Alterations of copper metabolism in other tissues are also correlated with potentiation of morphine analgesia and reversal of morphine tolerance with DβH inhibitors as well as effects of antagonist treatment and withdrawal. Treatment with DDC lowered plasma copper and ceruloplasmin levels to 75% of normal [209]. Morphine given alone or with DDC produced this same lowering of plasma copper, while levallorphan blocked the morphine-induced decrease in plasma copper and, if given alone, levallorphan increased plasma copper to 170% of normal [204]. Acute and chronic morphine tolerance also produced a 200% increase in plasma copper and ceruloplasmin levels and withdrawal caused a lowering to 50% of normal over a 1 week period [204]. When DDC was given to acute or chronic morphine-tolerant mice or rats, plasma copper levels fell to between 50% and 75% of normal within 0.5–1 h with the elimination of tolerance [204, 209]. Morphine withdrawal following acute tolerance caused a return to normal plasma copper and ceruloplasmin levels and an increase in copper content of the liver, the principal copper storage organ and site of ceruloplasmin synthesis. Tolerant mice treated with DDC also had elevated liver copper contents, while levallorphan-treated mice had normal liver copper contents [204]. Acute morphine tolerance also caused a 100% increase in biliary copper, while DDC treatment lowered biliary copper content to 25% of normal.

Established correlations between changes in copper content of brain tissues associated with DDC- or PTT-induced analgesia and potentiation of morphine analgesia led to demonstrations that treatment with inorganic copper salts produced analgesia and potentiated morphine analgesia which could be reversed by opioid antagonists [206, 210–212]. Iwata and Watanabe [206] originally demonstrated that $CuSO_4$ (63 μmol/kg) given i.p. produced analgesia in the tail-clip pain model. This dose of $CuSO_4$ following an s.c. injection of morphine (35 μmol/kg) produced a more prolonged and 4-fold greater analgesic response than morphine alone. Only a slight increase in analgesic activity was observed with this regimen in the hot-plate pain model. Stern [210] reported potentiation of morphine analgesia following i.p. administration of $CuCl_2$ (75 μmol/kg). Potentiation of morphine analgesia by $CuSO_4$ and $CuCl_2$ is consistent with the observation that $^{67}CuCl_2$ injected into the carotid artery was measured in the brain within seconds after injection and the amount crossing the blood-brain barrier was markedly increased by coadministration of amino acids [213]. Bhargava [211] subsequently demonstrated that intracerebral injection of 0.6 μmol $CuSO_4$/kg potentiated morphine analgesia in non-tolerant and morphine-tolerant mice. Jumping, but no

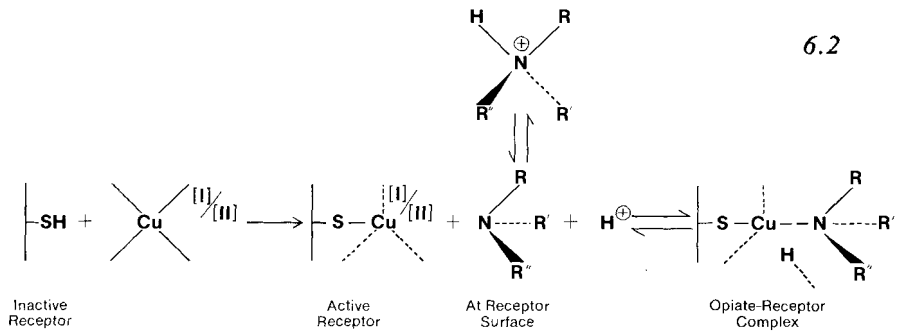
other abstinence symptoms, induced by naloxone was prevented when this dose of $CuSO_4$ was given to morphine-dependent mice. Finally, Marzullo and Hine [212] demonstrated that intracerebral (i.c.) injection of $CuCl_2$ (ED_{50} = 4.8 nmol) produced naloxone-reversible analgesia in the tail-flick pain model. Intracerebral treatment with 10–25 nmol dithiothreitol, but not its disulphide, reversed analgesia produced by $CuCl_2$ and also antagonized morphine analgesia.

The published literature supports the hypothesis that activation of opiate receptors is copper-dependent. DDC and PTT are analgesic and may potentiate morphine analgesia as a result of copper transport across the blood-brain barrier as lipophilic copper chelates. Inorganic copper salts are also analgesic and potentiate morphine analgesia, which may also be the result of lipophilic copper complex formation *in vivo*. Dithiothreitol, a copper-reducing and -complexing agent, reverses both $CuCl_2$ analgesia and morphine analgesia. Based upon these observations, it is hypothesized that bonding of opiates to opiate receptors is copper-dependent.



6.1

In 1954, Beckett and Casy [214] proposed an 'anionic' site on the opiate receptor to account for bonding of opiate agonists and antagonists which were recognized as having a cationic component at biological pH values as shown in *Scheme 6.1*. Subsequently, a great deal of research was done to increase



6.2

understanding of the structural requirements for opiate agonist and antagonist bonding to opiate receptors, while little has been done to characterize further the supposed 'anionic' site.

Observed analgesic activities of copper complexes and existing literature support the hypothesis that the originally proposed 'anionic' site may be better represented as cationic due to the presence of an atom of copper bonded to the proposed 'essential' thiol group [215–217] of the opiate receptor, with a hitherto unknown oxidation state, Cu(I) or Cu(II), and with as yet unknown additional coordinate-covalent bonding to the receptor. This hypothesis utilizes the 'essential' thiol group on the 'inactive' opiate receptor and proposes activation of this inactive form, by bonding with copper. Subsequent bonding of the opiate to the proposed active form of the receptor may lead to receptor stimulation as shown in *Scheme 6.2*.

Activation of inactive receptors by copper is consistent with analgesia produced by copper complexes of DDC or PTT formed *in vivo* following treatment with DDC or PTT, analgesia produced by copper complexes formed *in vivo* following treatment with $CuCl_2$ or $CuSO_4$, and analgesia produced by treatment with copper complexes. Following receptor activation with copper and in the absence of treatment with an exogenous opioid analgesic, the ensuing analgesia may be due to endogenous dynorphins, endorphins or enkephalins. This hypothesis leaves open the question as to whether or not receptor activation and analgesia can occur as a direct result of ligand exchange once the copper complex reaches the aporeceptor. A copper requirement for receptor activation is also proposed as consistent with observations that DDC and PTT (that is, their copper complexes formed *in vivo*), and inorganic salts of copper (that is, copper complexes formed *in vivo*), potentiate morphine analgesia and reverse morphine tolerance. In the former case, activation of more receptors by copper complexes may facilitate analgesia with smaller doses of morphine. In the latter case, if all activated receptors are occupied by morphine in the tolerant state, then activation of additional receptors with copper could facilitate analgesia following treatment of the morphine-tolerant animal with morphine.

Quaternary salts of opiate agonists and antagonists are generally less active than the parent non-quaternized agonist or antagonist. This may be accounted for with this model as resulting from the lack of a bonding interaction at the proposed cationic site with bonding only to the postulated cavity and flat sites of receptors [214]. In any case, a decrease in receptor bonding at the cationic site is proposed as consistent with the decrease in activity found with quaternized agonists and antagonists.

It is not proposed that opioid analgesics form copper complexes *in vivo* and

that these complexes facilitate transport of copper into the CNS, since their functional groups are not likely to form stable coordinate-covalent complexes capable of competing with other ligands in biological systems, which are capable of forming more stable complexes. The possibility does exist, however, that kyotorphine, dynorphins, enkephalins or endorphins can form stable copper complexes in the CNS which may be capable of both receptor activation and blocking nociceptive stimuli in their production of analgesia. The Cu(II)(methionine-enkephalin) and Cu(II)(leucine-enkephalin) complexes have been shown to be slightly more analgesic than their parent peptides without copper [218].

The appearance of copper in the CNS in association with the administration of DDC, morphine, inorganic copper, or a combination of these agents may indicate a role for copper in modulating biochemical processes associated with neural depolarization and pain perception. Rajan, Colburn and Davis [219] have suggested that copper may be involved in monoamine storage and transport as ternary copper chelates of catecholamines and ATP. Data in *Table 6.7* show modulation of both adenylate cyclase and phosphodiesterase activity with inorganic copper compounds and support the suggestion that copper may have a role in regulating these activities [220–223]. It appears that phosphodiesterase activity is less sensitive than adenylate cyclase activity to inhibitory effects of inorganic copper. In addition, amounts of copper added

Table 6.7. INHIBITION OF ADENYLATE CYCLASE AND PHOSPHODIESTERASE ACTIVITIES WITH COPPER CHLORIDE OR SULPHATE [220–223]

Enzyme	*% Inhibition*	*Cu salt concentration (μmol)*	*Ref.*
Adenylate cyclase	50	1.0	218
	50	2.6	213
	90	100	215
Phosphodiesterase			
Hydrolysis of cAMP	35	0.1 to 1	213
	30[a]	100	216
	30[b]	100	216
	94	2000	214
Hydrolysis of cGMP	24[a]	100	216
	17[b]	100	216

[a] Without phosphodiesterase activator.
[b] With a saturating amount of phosphodiesterase activator.

are very large when compared with the analgesic ED_{50} dose of inorganic copper (4.8 nmol) when it is given i.c. [212]. These results are consistent with those of Lux and Globus [224] as well as Sakai and Iwata, as pointed out by Iwata, Watanabe and Matsui [205], showing inhibition of postsynaptic spinal motor neurons with $CuSO_4$ and opioid analgesics, respectively. It is also of interest that concentrations of 1–10 mmol dithiothreitol added to $CuSO_4$-inhibited preparations of adenylate cyclase increased adenylate cyclase activity to 70% above noninhibited control values [205]. Dithiothreitol disulphide and other disulphides had no effect on $CuSO_4$-inhibited adenylate cyclase activity, which supports the suggestion that copper may be reacting with a thiol group required for adenylate cyclase activity [205]. Opioid analgesics and endogenous peptides may also have a role in potentiating this copper-dependent modulation of adenylate cyclase activity.

Analgesic activities of copper complexes, as well as their antipyretic and anti-inflammatory activities, offer some explanation for their remarkably successful treatments of arthritic and other degenerative diseases of man [138].

THERAPEUTIC EFFECTIVENESS OF COPPER COMPLEXES IN TREATMENT OF ARTHRITIC AND OTHER DEGENERATIVE DISEASES

Uses of copper and its compounds to treat infections, inflammations, arthritidies, tumour and epilepsy, and to promote wound and bone healing are recorded in ancient Egyptian papyri (2600 and 2200 B.C.) and in Greek, Roman, Aztec, Hindu and Persian writings [138]. It is likely that European uses of copper were based upon recorded Persian treatments. German and French publications report the successful use of copper oxide in treating tuberculosis during the 1920's and 1930's [225]. By1940 physicians and chemists had collaborated in the development of additional copper complexes found to be useful as antitubercular agents, and one of them, Cu(I)[Na-3-(allylthiouredo-1-benzoate] (allocupreide sodium, Cupralene, 19% Cu) [94], was suggested to be superior to the then current gold therapy of tuberculosis [226–228].

In 1941, Fenz used this antitubercular drug to treat rheumatoid arthritis (RA), since it was thought that arthritis also had an infectious etiology. He found that intravenous doses of 0.01–0.1 g (2–20 mg of Cu) gave modest results, but exceptional results were claimed with some patients given 0.2 g (40 mg of Cu) per injection three times per week with a limit of 2.5–6 g (475–1140 mg of Cu) per treatment course [229, 230]. Fenz also reported an additional antianaemia effect on anaemias associated with RA which was consistent with observations reported by others, who had found that anaemic

or chlorotic girls who went to work in a copper mine were soon relieved of their anaemia [229]. This is consistent with what is now known to be a copper-dependent iron-mobilization process required for haemoglobin synthesis [8, 231]. Fenz also reported that toxic side effects, such as those that occur relatively often with gold therapy, were not observed with copper therapy.

Forestier, a renowned French rheumatologist, who was the first to report the successful use of gold therapy in the treatment of arthritic diseases [232] also recognized that gold therapy was associated with a high incidence of toxic reactions and intolerance, and in his search for drugs with less toxicity he began to use allocupreide in 1942. In 1944 he published preliminary results of his studies which were patterned after his 20-year clinical experience with the use of gold compounds. His first group consisted of 33 RA, 4 ankylosing spondylitic and 6 osteoarthritic patients [233]. Of these, 45% were reported to have had 'very good' results, 20% had 'favourable' results and 35% had

Table 6.8. RESPONSE OF PATIENTS WITH DIFFERENT DURATIONS OF RHEUMATOID DISEASES FOLLOWING ALLOCUPREIDE TREATMENT [94]

	Patients	*Clinical results*[a]			
		very good	*good*	*moderate*	*none*
Rheumatoid arthritis (less than 1 yr duration)	13	5(38%)	7(54%)		1(8%)
Rheumatoid arthritis (more than 1 yr duration)	23	1(4%)	8(35%)	9(39%)	5(22%)

[a] No definition of classification given.

'moderate improvement,' which was consistent with the results obtained by Fenz [229, 230]. In addition, Forestier and Certonciny [234] found that increasing the dose to between 0.25 and 0.5 g (47.5–95 mg of Cu) per intravenous injection and increasing the duration of therapy as well as total dose per series to between 2 and 5 g (380–950 mg of Cu) increased the percentage of RA patients receiving effective therapy, as shown in *Table 6.8.*

As might be expected, more patients with RA had 'very good' responses if the duration of their disease was less than 1 year. There were also fewer patients in the 'moderate' and 'no effect' groups when the disease was of shorter duration. All patients with chronic polyarticular synovitis were favourably affected, but none of their responses was rated as 'very good.' No beneficial effect was observed in the ankylosing spondylitis, acute articular

rheumatism or monoarthritis disease categories. In contrast to these, a 'good' response was achieved by the one patient who had staphlococcal spondylitis.

As a result of their continuing search for more effective therapy Forestier, Jacqueline and Lenoir [235] reported that a new copper complex, Cu(II)[8-hydroxyquinoline di(diethylammonium sulphonate)]$_2$(cuproxoline, Dicuprene or Cuprimyl), was more effective than allocupreide. Although this compound had been found by Michez and Oretegal [236] to be less effective orally, Forestier and his colleagues considered cuproxoline to be superior to allocupreide because it was less irritating and could be given by both intravenous and intramuscular routes of administration.

Cuproxoline (6.5% Cu) was given intramuscularly or intravenously at a dose of 0.5–1.0 g two or three times a week (32.5–65 mg of Cu) with a total of 6–12 g (390–780 mg of Cu) per series. As with gold therapy, this copper compound was given in a series of injections with rest periods between series. Intervals between series were not to exceed 1 month between the first two series and 2–3 months between subsequent series. Any one series of injections was not sufficient to ensure that all signs of active arthritis disappeared and ESR returned to normal.

Table 6.9. RESPONSE OF PATIENTS WITH DIFFERENT RHEUMATOID DISEASES FOLLOWING DICUPRENE TREATMENT [94]

	Patients	*Clinical results*[a]			
		very good	*good*	*moderate*	*none*
Rheumatoid arthritis (less than 1 yr duration): copper therapy used initially	4		4(100%)		
copper therapy after gold therapy	1		1(100%)		
Rheumatoid arthritis (more than 1 yr duration): copper therapy used initially	13	3(23%)	7(54%)	2(15%)	1(8%)
copper therapy after gold therapy	18	2(10%)	9(50%)	2(10%)	5(30%)
Gonococcal arthritis	4		2(50%)	1(25%)	1(25%)
Chronic polyarticular gout	3	2(67%)	1(33%)		
Chronic polyarticular synovitis	4	2(50%)	1(25%)	1(25%)	
Ankylosing spondylitis	3	2(67%)			1(33%)
Disseminated spondylitis	1	1(100%)			
Monoarthritis	2	1(50%)	1(50%)		

[a] No definition of classification given.

As shown in *Table 6.9*, cuproxoline therapy also produced beneficial results when it was used to treat RA which had been active for less than a year, even if the patient had become resistant to gold therapy.

Patients who had RA for longer than a year were also successfully treated with cuproxoline. Unlike allocupreide, this drug was also found to be effective in patients with ankylosing spondylitis and disseminated spondylitis. In addition, Christin, in a personal communication to Forestier and Certonciny [234], reported that patients with both RA and psoriasis received simultaneous amelioration of both articular and skin lesions when treated with cuproxoline.

Forestier's comparison of results he and his colleagues had obtained with copper and gold therapy of RA and other degenerative diseases demonstrated that copper therapy was superior to gold therapy [237–240]. With RA of less than 1 year in duration, copper therapy (particularly allocupreide) was more effective and less toxic than gold therapy. When gold therapy was tolerated, it was preferred for patients who had RA for more than 1 year in duration. However, copper complexes were far superior to gold therapy in treating chronic polyarticular synovitis with effusion [238]. This definite clinical entity associated with a high ESR and anaemia was found to be resistant to gold therapy. Effusions cleared fully or partially in 80% of these patients after two or three series of injections. Treatment of polyarticular gout was successful in 37 patients [239]. Twenty-one patients given intramuscular injections for two or three series enjoyed remarkably rapid elimination of signs of gouty polyarthritis and eight others were ameliorated with four or five series. In eight other more recent cases, intravenous or intramuscular therapy was successful when continued to remission. Their first attempts with ankylosing spondylitis were less successful, but increasing the dose and keeping their patients on a very strict regime of injections gave improved results. Psoriasis arthropathica generally responded rapidly to copper therapy, which was far superior to gold therapy and brought about astonishing improvements in both skin and joint lesions [237]. Beneficial use of copper therapy in infectious arthritis of known or unknown origin or in rheumatic fever was less certain [237]. A decrease in ESR was found to correspond with clinical improvement over a period of 2 or 3 months.

Copper therapy administered according to recommended doses did not as a rule cause untoward reactions. However, 'shock' occurred after an intravenous injection when the compound had not been completely dissolved. Slight transitory malaise and nausea were occasionally observed. Three cases of slight jaundice were observed, but there was no skin yellowing, albuminuria or thrombocytopenia. Patient tolerance of copper therapy was found to be superior to that of gold therapy.

In 1950 Forestier, Certonciny and Jacqueline [240] published detailed results of their study demonstrating that copper complexes were effective in treating patients who were intolerant of or resistant to gold therapy (*Table 6.10*).

Following institution of therapy, the onset of improvement was immediate, definite and progressive in 20 patients, slow and progressive in 14 patients, and delayed in one patient. Twelve patients who were in this group were followed for over 2 years and showed no relapse. It was felt that this period was long enough to make it unlikely that these were spontaneous remissions. The ESR returned to normal in 13 patients with RA of less than 1 year duration. Fifty-one of 59 patients experienced no ill effect attributable to the medication. Only minor side-effects such as malaise, nausea and slight albuminuria were noted in 8 patients. No sign of marrow depression was noted. Copper compounds used to continue therapy in patients who were resistant to or intolerant of gold therapy were found to be advantageous, since they could be used without ill effects in the event of gold therapy-induced dermatitides (rashes, stomatitis) or nephritis (albuminuria).

In 1950, Tyson, Holmes and Ragan published their evaluation of allocupreide in what were described as 27 typical RA patients [241]. While they achieved modest successes in therapy, they failed to follow Forestier's protocol accurately, and what appeared to be iatrogenic toxic effects resulted which may have been due in part to drug decomposition [94].

Table 6.10. RESULTS OF TREATMENT OF PATIENTS WITH RHEUMATOID ARTHRITIS WHO WERE RESISTANT TO OR INTOLERANT OF GOLD THERAPY [94]

	Patients	*Clinical results*[a]			
		greatly improved	*improved*	*Clinically improved*[a]	*no improvement*
Less than 1 yr duration: copper used first	18	6(33%)	7(39%)	1(11%)	3(17%)
Over 1 yr duration: copper used first	15	3(20%)	3(20%)	1(7%)	8(53%)
Copper used after resistance to gold therapy	14	1(7%)	2(14%)	3(21%)	8(58%)
Copper used after intolerance to gold therapy	12	2(17%)	4(33%)	1(8%)	5(42%)

[a] Improved – persistent high sedimentation rate. No other classification given.

Table 6.11. RESULTS OF COPPER THERAPY OF RHEUMATOID AND OTHER DEGENERATIVE DISEASES [94]

Diseases in various classes and stages	*Drug[b] Number of patients*	*Clinical results[c]*			
		grade I	*grade II*	*grade III*	*grade IV*
Rheumatoid arthritis[a]	C-27, A-4	2(6%)	4(13%)	9(29%)	16(52%)
Chronic gouty arthritis	C-17, A-1	3(16%)	5(28%)	5(28%)	5(28%)
Psoriasis with arthritis	C-8, A-1	1(10%)	4(45%)		4(45%)
Ankylosing spondylitis	C-3	1(33%)		1(33%)	1(33%)
Reiter's syndrome	C-3	3(100%)			
Lupus erythematosus	C-1			1(100%)	

[a] Disease progressed in only one patient.

[b] C, Cuproxoline; A, Allocupreide.

[c] Grade I: Complete remission. Grade II: Major improvement in which the sedimentation rate may still be moderately elevated but in which there is absence of systemic signs of activity such as leukocytosis and fever. There are no signs of joint inflammation, although minimal changes due to irreversible capsular thickening may be present and no new rheumatoid process is present. Grade III: Minor improvement not considered significant, since the disease has the inherent capability of variation in signs and symptoms. In this grade, however, are all those who had slight improvement prevents them from being Grade IV and is yet so small that they cannot be Grade II. Grade IV: No therapeutic response.

In the following year, Kuzell, Schaffarzick, Mankle and Gardner [242] published their study of copper therapy in a variety of rheumatoid and degenerative diseases. The purpose of their clinical study was to evaluate cuproxoline and allocupreide in patients who had RA, RA with psoriasis, Reiter's syndrome, ankylosing spondylitis, and chronic gouty arthritis, as shown in *Table 6.11.*

Patients with RA were given a total dose of 2.2–17.0 g of allocupreide intravenously or cuproxoline intravenously or intramuscularly in divided doses two or three times weekly and classified according to extent of arthritis and degree of therapeutic response, according to the system outlined by Steinbrocker, Trager and Batterman [243] and the American Rheumatology Association. Of the 31 RA patients treated, only 6 reached Grades I and II. Therefore, only 20% appreciably improved and that was considered to be less than satisfactory or apparently without benefit. Five patients, or 55% of those treated, who had less severe or early stage psoriasis with RA responded with

Grade I or II improvement without skin-lesion progression or exacerbation. Slightly more improvement in these patients than those arthritics without psoriasis was suggested to merit further study. Two of the three patients with ankylosing spondylitis showed responses of Grades I and III. However, these patients received other drugs in addition to copper complexes, which prevented any conclusion concerning the efficacy of copper therapy in these cases. Three patients with Reiter's syndrome were 'apparently cured' with cuproxoline. Positive cultures of pleuropneumonia-like organisms were obtained from conjunctiva and, in the male form, penile sores and urethral discharge. Only a limited Grade III response was observed with cuproxoline for one patient with disseminated lupus erythematosis. Of 18 patients with chronic gouty arthritis 13 had a Grade I, II or III response to copper therapy which was also viewed as worthy of further study.

However, although this does seem to be inconsistent, these authors concluded that, based upon these limited successes, copper complexes were considered to be therapeutically ineffective. This conclusion was based upon the supposition that the newly discovered corticoids were going to cure arthritic diseases.

According to these authors, the toxicity of these substances was slight, infrequent, and of a mild transitory nature in contrast to toxicities observed by others [241]. The total number of toxic reactions noted in relation to the large number of injections given to these groups of patients indicated a low order of toxicity. No change was noted in either blood counts or urinanalyses. They also noted the same garlic-like breath odour associated with allocupreide therapy as reported earlier [241], which has been suggested to have been due to decomposition of allocupreide [94].

Consistent with the report of Forestier and Certonciny [239] that the LD_{50} values determined in rats for allocupreide and cuproxoline were 160 mg/kg and 120 mg/kg, respectively, rat LD_{50} values were found to be 160 mg/kg (intraperitoneal) and 375 mg/kg (intramuscular) for allocupreide and 126 mg/kg following intramuscular administration of cuproxoline [242].

Although allocupreide and cuproxoline were shown to be effective and in many instances superior to gold therapy of rheumatoid and other degenerative diseases, nothing more was published concerning these or the other copper complex drugs after 1955. There appear to be at least two reasons for this. First, there was little or no generally recognized requirement for copper nor any real appreciation for essential metalloelement metabolism in any biological system, and there was no source of such information as there is today [1, 39, 40]. A second reason may have been the discovery of hydrocortisone, which was thought to be the 'cure' for all rheumatic disease. Just prior to 1949

and subsequent to that time, hydrocortisone consumed the attention of nearly all researchers and physicians interested in treatment of rheumatic diseases.

Fortunately, Werner Hangarter, a German physician who was Head of Medicine at the University of Kiel, was just beginning his research with a new copper preparation, Permalon, in 1950. Permalon was a Cu(II)-salicylate-containing preparation developed by Hangarter in collaboration with Reiser of the Albert Chemical Company.

The following is an account of Hangarter's work at Bad Oldesloe Hospital, which continued until 1971 when he retired and the manufacture of Permalon was discontinued for economic reasons. This account comes from detailed reviews published elsewhere [41, 94, 244] and is presented here for the benefit of readers of this chapter.

Historically, the therapeutic potential of copper for the treatment of rheumatic diseases was first recognized by Hangarter in 1939 when he learned that Finnish copper miners were unaffected by rheumatism as long as they stayed with the mining industry [245]. This was particularly striking, since rheumatism was a widespread disease in Finland, and workers in other industries and other towns had more rheumatic disease than these copper miners. Furthermore, it was stated by the mine company's physician that purulent infections occurring either spontaneously or as a result of injuries were seldom observed in copper miners as compared to workers in other industries in Finland.

Therapeutic results with copper alone were found by Hangarter to be comparable with those of chrysotherapy, although copper treatment was associated with considerably fewer side effects [246]. One ampoule of Permalon contained 20 ml of an aqueous solution containing 2.0 g (12.5 mM) of sodium salicylate and 2.5 mg (39 mM, 2.5 mg of Cu) of copper, added as the chloride. It is likely that this solution contained copper complex(es) of the added salicylate.

From 1950 to 1954, Permalon was administered by intravenous injection. Daily injections over an average period of only 8–14 days produced the best results. In cases of obvious therapeutic success, this period of medication was followed by one or several days without treatment. A sudden discontinuation was not advised, since prolonged administration produced a better and longer-lasting effect.

Hangarter's first experiences with Permalon resulted in remission of fever, alleviation of pain, increased mobility, inhibition of exudation of joint effusions, and a decrease in the ESR in various stages [243] of rheumatic diseases [245]. His clinical results demonstrated therapeutic success, as shown in *Table 6.12*.

Table 6.12. EARLY RESULTS OF THERAPY OF SOME RHEUMATOID AND DEGENERATIVE DISEASES WITH PERMALON [94]

Type of disease	*Number of patients*	*Clinical results*		
		symptom-free[a]	*improved*[b]	*unaffected*[c]
Acute rheumatic fever	22	11(100%)		
Rheumatoid arthritis	27	13(48%)	13(48%)	1(4%)
Erythema nodosum	2	2(100%)		
Sciatica, lumbago	5	5(100%)		

[a] Symptom-free: The absence of articular inflammation, disappearance of nonarticular inflammation, return of articular mobility – deformation only as a result of irreversible changes, normal ESR, no radiological evidence of progression. Free from pain and fever.

[b] Improved: ESR was still elevated, articular swelling though only slight still present, disturbances in articular mobility with only little sign of activity still evident, no increase in deformities, no radiological evidence of progression. Arthralgia only occasionally. No fever.

[c] Unaffected: General condition unchanged, painful, no change in inflammatory signs, radiological evidence of progression, elevated ESR, restricted mobility, deformation and fever of varying degrees, but not significantly decreased for evaluation as improved.

Pain, reddening and swelling of affected joints in acute rheumatic fever (ARF) were markedly decreased after a single injection of one ampoule of Permalon (20 ml), and patients were completely free from pain after one or two additional administrations. The loss of pain was accompanied by a drop in temperature in most patients. Mean articular mobility was improved due to a rapid regression of swellings after one or two intravenous injections. Erythrocyte sedimentation rates decreased more slowly, although constantly, and treatment was continued until normal ESR values were achieved. No relapses were recorded during the unspecified observation period. On an average, 20 injections were necessary to normalize all clinical and serological values. Results of this therapy were comparable with those obtained with massive doses of oral salicylate, but were not associated with its toxic side-effects or intolerance.

Intravenous therapy of rheumatic carditis with Permalon also gave remarkable results. After transient, highly inflammatory acute articular episodes accompanied by high fever, a typical endocarditis or myocarditis had developed in 11 patients. In these severe cases, all signs of articular inflammation completely subsided after three or four injections at intervals of 12 h. Cardiovascular performance was restored both from the clinical and electrocardiographic points of view. Symptoms of endocarditis also diminished from day to day. Contrary to the favourable objective clinical course of the disease, ESR

values improved only gradually. However, treatment was continued until ESR values were normalized.

The broadest clinical experience with Permalon therapy was gained in the treatment of RA. Many patients had been previously ineffectively treated with a variety of antirheumatic drugs, including salicylates, gold and corticoids.

Patients with RA responded to Permalon in what was described as a step-by-step fashion. The initial effect following the first injection was alleviation of pain. This rapid relief from pain was parallelled by remissions of fever and improved articular mobility, even in patients with severe deformities. A regression of articular swellings and extensive joint effusions were also observed early with Permalon therapy. Erythrocyte sedimentation rates were also characterized as dropping slowly but steadily. Even though the objective clinical picture indicated rapid and complete remission, ESR declined only gradually. In many cases a complete normalization of the ESR was observed only after discharge from hospital at out-patient follow-up evaluations. These results were pointed out as having been confirmed by Fahndrich [247] and Broglie [248]. Subsequent to the Hangarter and Lubke [245] report, intravenous Permalon therapy was reported to be effective in treating osteoarthroses by Schoger [249].

Therapeutic results with two patients suffering from classical erythema nodosum accompanied by very painful arthralgia served as a particularly significant criterion for this therapy, since experience had shown that this type of rheumatic disease is often very resistant to treatment. After two or three injections these patients were completely free of pain. At the same time, body and local temperatures dropped to normal along with an astonishingly rapid absorption of skin infiltrates. After 12 days of Permalon therapy ESR was normal. No relapses were recorded during the observation period and complications were not observed. Compared with oral sodium salicylate treatment (not given at the same time), Permalon was suggested to be much more effective and rapidly acting.

Rheumatic neuritis (sciatica and facial) also responded to Permalon therapy with rapid alleviation of pain. However, as far as motor impairment and duration of action were concerned, Permalon's effects did not differ from those of other therapeutic methods.

It is of interest to point out here that Hangarter had recognized the importance of copper in his preparation of Cu(II)-(salicylate). It was known that in order to achieve the same therapeutic success with only intravenous salicylate therapy, a serum level of at least 25 mg/100 ml was necessary. To achieve this concentration of unbound salicylate, these patients would have had to have been given more than 12 g of salicylic acid, six Permalon injections, divided

into equal administrations throughout a 24 h period. Since that much Permalon had not been given and the salicylate level in blood reached a peak value of only 20–24 mg/100 ml and dropped to 5–8 mg/100 ml within 24 h, a level much lower than the recognized therapeutically effective level of salicylic acid, he had evidence that Permalon's effect was not due to salicylic acid alone. It now seems reasonable to suggest that the marked therapeutic efficacy of Permalon was due to the presence of a Cu(II)-(salicylate) complex or complexes in this preparation.

It was striking that no systemic toxic or noxious side-effects were observed, even on long-term intravenous administration of Permalon. Analyses of blood components and kidney and liver functions (thymol test, Takata-Ara, Weltmann, and elimination of bile pigments) gave no evidence of pathological changes or reactions. Blood serum levels of all components remained normal. Tolerance was good even when two injections per day were given. It is particularly significant that gastrointestinal disturbances and cerebral toxic reactions, which were investigated, were not found. A few patients experienced transient sciatic pain during intravenous injection of the first Permalon preparation, but this effect was no longer observed after subsequent pharmaceutical development. (I was not able to determine the nature of this formulation change.) Occasionally intravenous administration of the new formulation was accompanied by pain or transient injection-site reddening if therapy was long-term, veins were in poor condition, or the 20 ml ampoule was injected too rapidly.

To overcome this irritation, Permalon was subsequently administered to all patients by intravenous infusion. This mode of administration achieved equally good results with fewer treatments and without causing venous irritation and it was possible to give much higher doses. All patients received 500 ml of physiological saline, containing 3 to 4 ampoules of Permalon per infusion. Infusion of 3 to 4 ampoules represented 6.0 to 8.0 g of sodium salicylate and 7.5 to 10.0 mg of copper. In order to avoid initial irritation with slow infusions, a 2 ml ampoule containing 0.4 mg of novacaine was routinely added to the infusion solution. This dose of novacaine was much less than the usual therapeutic dose of 4.0 mg/kg of body weight, according to Fellinger and Schmid [250] and was not considered to be a systemic analgesic dose. The average duration of remission for these patients was 3 years (Hangarter, personal communication).

The number of infusions necessary to achieve therapeutic success again depended upon onset and degree of disease. This was similar to results obtained with allocupreide and cuproxoline, which were found to be more effective in diseases of shorter duration. However, Permalon was effective in

diseases of all durations when given in the prescribed manner. In general, six to eight infusions (45 to 80 mg of Cu) at intervals of 2–4 days sufficed. These infusions were well tolerated despite the high dosage level. A transient nausea accompanied by tinnitus, a common side-effect of salicylate therapy of RA, was observed, but this was considered to be a minor effect in view of the rapid regression of usually severe symptoms and signs of disease.

Absence of gastrointestinal disturbances, usually observed at much lower salicylic acid doses, was again striking. With infusion therapy, the blood picture, kidney and liver functions, blood sugar, serum electrophoresis, electrolyte metabolism, ECG and rheumatic serology were evaluated. No pathological changes or abnormal reactions were found. Nor were there any cerebral, respiratory, or circulatory toxicities observed.

With experience gained from 1954 to 1971, Hangarter was able to draw some comparisons between results obtained with Permalon therapy and commonly used antirheumatic agents such as nonsteroidal drugs, cortisone preparations, gold salts, as well as antimalarial drugs. In general, Permalon therapy was superior to all of these. Clinical results of intravenous infusion therapy obtained from 1952 to 1971 [246] are presented in *Table 6.13*. These results with Permalon infusions corresponded to very successful clinical re-

Table 6.13. RESULTS OBTAINED BY HANGARTER WITH PERMALON THERAPY [94]

Disease	*Number of patients*	*Clinical results*[a]			
		symptom-free	*improved*	*slightly improved*	*un-changed*
Acute rheumatic fever	78	78(100%)			
Rheumatoid arthritis	620	403(65%)	143(23%)		74(12%)
Cervical spine-shoulder and lumbar spine syndromes	162	95(57%)	52(32%)		18(11%)
Sciatica[b]					
without lumbar involvement	120	76(63%)	38(32%)	6(5%)	
with lumbar involvement	160	95(59%)	39(24%)	10(6%)	16(11%)

[a] See footnotes in *Table 6.5* for the definition of clinical result classifications for acute rheumatic fever, rheumatoid arthritis, and cervical spine-shoulder and lumbar spine syndromes.

[b] Symptom-free: Disappearance of (subjective) symptoms; Lasague's sign negative, normal reflexes with equal quality on both sides, no distrubances in sensitivity, no tenderness on pressure, and mobility restored. Improved: Not completely relieved from (subjective) symptoms or persistence of one or several symptoms listed in the symptom free classification, yet no longer any impairment of mobility. Slightly improved: Impairment of walking ability still demonstrable, though only moderate, with persistence of one or several symptoms. Unchanged: No response to treatment at all.

sults previously described for those patients treated with daily intravenous injections of a single 20 ml ampoule of Permalon. With infusion therapy, 78 ARF patients (100%) experienced an almost immediate subsidence of acute symptoms after one or occasionally after two or three infusions. Average duration of treatment was only 14–18 days. Assessment of infusion therapy was also based on criteria provided by the American Rheumatism Association and those published in the Journal of the American Medical Association [243]. Therapy produced remission of fever, increased articular mobility, decreased swellings, as well as normalization of ESR and the absence or disappearance of cardiac manifestations.

From 1954 to 1971, 620 patients in all stages and durations of RA received Permalon by intravenous infusion. The number of infusions required for therapeutic success was dependent on the previous course, onset and severity of disease. On an average, it ranged from six to a maximum of ten infusions. Patients having RA, as well as aggressive forms of polyarthritis, also progressed to a symptom-free classification in a step-by-step manner. The initial effect, evident after one or two infusions, was a marked alleviation of pain and improved mobility. This rapid relief from pain and improved articular mobility was readily seen in cases of severe deformities. Just as in ARF, infusions brought about a remission of fever and a constant regression of articular swellings and extensive articular exudations associated with RA. Erythrocyte sedimentation rates, which were the most important indicator of all subjective pathological processes and mesenchymal damage, were characterized by a slow and steady drop parallelling the decrease in rheumatic serology. Even though the objective clinical picture indicated very rapid complete remission, elevated ESR decreased only gradually. In many cases, a normal ESR was reached only after hospital discharge in outpatient follow-up studies. Latex tests remained positive longer than elevated ESR values. No serious toxic disturbances were recorded in association with this higher dose of Permalon. However, there was a toxic dose limit where nausea and tinnitus appeared, but in view of the rapid alleviation of pain, these toxicities were still considered to be minor side-effects by these patients.

Patients with cervical spine-shoulder syndrome, including shoulder bursitis (frozen shoulder, calcareous tendonitis and Duplay's disease) as well as lumbar spine syndrome, were also successfully treated with Permalon. These patients were generally treated with Permalon infusions as described for patients with RA. However, when these patients suffered from marked impairment of mobility accompanied by severe nocturnal pain, they were given an average of 4–8 infusions (40 to 80 mg of Cu) per day at intervals of 2–3 days. From 1970 to 1971 these patients received an additional treatment

of two tablets of Colfarit (microcapsulated salicylic acid, Bayer Leverkusen) 3–4 times daily on days between individual infusions in association with follow-up treatment which also included physiotherapy. Of the 162 patients who had undergone this therapy (primarily Permalon), 92 became symptom-free, 52 improved with complete relief from pain but had a persistent slight impairment of mobility, while only 18 experienced no beneficial effect.

From 1956 to 1971, 280 patients who had the diagnosis of sciatica syndrome with and without spinal changes underwent in-patient treatment with Permalon. Sciatica syndrome included a wide range of diseases, not necessarily recognized as rheumatic in origin. Some of these were genuine sciatic neuritis, sciatic neuralgia of different focal or toxic origins, and radicular nerve irritation as a result of vertebral or intervertebral disc changes. Consequently, this syndrome also included symptoms which were not believed to be rheumatic. Most patients were seriously affected and suffered from long-lasting pain, particularly nocturnal pain, and considerable impairment of mobility with regard to walking and changing their position. In these cases, objective symptoms were accompanied by true neurological and trophic disturbances as well as highly acute sciatica associated with severe shooting lumbagoid pain. Some patients presented with a highly positive Lasegue's sign, without reflexes or sensitivity to stimuli. With a majority of sciatic cases, there were no signs of neuritis as such and the etiology appeared to be due to a slipped disc. Most patients included in this Permalon study had already undergone several years of unsuccessful orthopaedic and conventional anti-rheumatic therapy. Therapy designed to treat neuritis and neuralgia was also unsuccessful.

Depending on duration and severity of disease, these sciatic patients received between six and eight intravenous infusions of Permalon (60–80 mg of Cu) every 3–4 days. In spite of the above-mentioned diverse etiology of these diseases, Permalon therapy was very effective. Even those who had undergone previous unsuccessful treatment with conventional preparations were rapidly and persistently relieved from pain which was accompanied by an overall alleviation of their condition. Far advanced abnormalities in posture as a result of spinal or neurological lesions were improved. Impairment of mobility regarding walking and an inability to change one's position subsided within a short period. *Spinal scoliosis, which may have been positional, was corrected!* Neurological symptoms provoked by the disease, absence of or differences in reflexes, a positive Lasegue's sign, disturbances in sensitivity, and trophisms were normalized very rapidly. Twelve of the 16 patients who were therapy-resistant had serious slipped disc problems which required neurosurgical repair.

Apart from tinnitus, sweating and transient nausea, no serious toxic disturbances were observed. Blood chemistry, kidney and liver functions, serum electrophoresis, and electrolyte metabolism revealed no pathological changes or abnormal clinico-chemical reactions.

All of the above raises an interesting question: Could the above diseases have been more effectively treated and/or prevented if copper complex therapy had been given earlier in their course?

Renewed interest in the use of copper complexes has led to a number of recent developments. Pratt, Omdahl and Sorenson [251] supplemented diets of seven adult patients with back pain with either placebo or 71.5 mg of Cu(II)(gluconate)$_2$ (10 mg of Cu)/day for 12 weeks and found no significant changes in hematocrit, corpuscular volume, serum cholesterol, serum triacylglycerols, SGOT, serum alkaline phosphatase, GGT or serum LDH in either group, but only one individual in each group benefited with relief of back pain with this supplementation [252]. Since Cu(II)(gluconate)$_2$ is water-soluble and likely to have been easily excreted, its low anti-inflammatory activity in man is consistent with little or no anti-inflammatory activity for water-soluble copper-containing compounds against kaolin paw oedema [163].

Patents have issued for the use of amino-acid complexes [253], copper-metallothioneins [254], compositions of copper compounds mixed with fatty acids [253], copper complexes of D-penicillamine, alkylcysteines [256, 257] and copper complexes of fatty acids alone or mixed with metallic copper [258]. Compositions of copper compounds mixed with fatty acids were also claimed to be useful in the treatment of other inflammatory disorders, including cardiovascular and thrombotic disorders, menstrual cycle disorders, diabetes, endometriosis, nutritional deficiencies and malignancies [255]. Scheinberg has also obtained a Food and Drug Administration approved Investigational New Drug application for the treatment of RA with the mixed-valence copper penicillamine complex (personal communication). Preparations of Cu(II)-(oleate) are currently being sold in Europe for topical treatment of RA and other inflammatory disorders under the trade names of Kupfer and Kupfer Forte, which contains fine particles of metallic copper [258].

Walker, Griffin and Keats, as pointed out in an earlier review, [84] have demonstrated the clinical efficacy of the copper bracelet in a single blind cross-over study. There have also been many clinical trials of Cu-Zn SOD. Unfortunately, the effectiveness of this enzyme in the treatment of inflammatory diseases has been disappointing. Its short half-life (6 min) and its inability to cross lipid cell membranes probably account for its lack of efficacy in treating arthritic and other inflammatory diseases.

GASTROINTESTINAL ULCERS

ALTERED COPPER METABOLISM IN ULCER DISEASES

There are no reports of altered copper metabolism in association with ulcer diseases. However, it is most likely that plasma copper is elevated in animal models of ulcer and in these human disease states. Remission of gastric and intestinal ulcers may, in part, be due to this physiological response, since copper complexes have antiulcer activity.

ANTIULCER ACTIVITIES OF COPPER COMPLEXES

The formation of copper complexes of non-steroidal anti-inflammatory drugs (NSAID) not only markedly improved their anti-inflammatory effect, but also eliminated their principal toxicity, ulcerogenicity, and produced compounds with very potent antiulcer activity in models of gastric and intestinal ulcer. Since it is well known that clinically used anti-arthritic drugs cause ulcers and gastrointestinal distress, the observation that copper complexes have antiulcer activity further distinguishes these compounds from their parent ligands as being safer and potentially much more therapeutically useful.

Original reports that many copper complexes were effective in preventing ulcers following oral dosing in the Shay and corticoid-induced ulcer models [22, 92] have been confirmed in the Shay and other animal models of gastric and intestinal ulcer. Copper complexes studied to date are listed in *Table 6.14*. From these studies, it seems to be true that nearly all copper-containing compounds are orally effective antiulcer agents. Although some are more potent than others, these relative potencies may depend upon the model of ulcer used. There is generally good agreement in comparisons of results obtained by various investigators. Some, however, have found gastric irritation using inappropriate mixtures of NSAIDs and inorganic copper salts or inappropriate interpretations of their experimental data [276, 277].

The lack of gastric irritation [270], the presence of antiulcer activity, and the enhanced anti-inflammatory activity of these complexes make this class of potentially useful antiarthritic drugs particularly promising, since the arthritic syndrome is likely to include gastric ulcers [259, 260].

Representative data from the original report of antiulcer activity for some NSAID copper complexes are presented in *Table 6.15*. Copper(II)$_2$(acetate)$_4$ had only very weak antiulcer activity, in contrast with copper complexes of the NSAIDs, which were found to be potent antiulcer agents. There are no more potent antiulcer agents in this model of ulcer. Comparison of the amount

Table 6.14. COPPER COMPOUNDS FOUND TO BE EFFECTIVE ANTIULCER AGENTS IN ANIMALS

Compound	*Reference*
Inorganic copper compounds and salvates	
Cu(I)Cl	273
$Cu(II)Cl_2$	152,170,261,262,274
Cu(II)O	273
$Cu(II)_2(acetate)_4$	22,261,262,297
$Cu(II)(pyridine)_2(acetate)_4$	22,296,297
$Cu(II)(pyridine)_2Cl_2$	22,297
$Cu(II)(imidazole)_2Cl_2$	274
$Cu(I)(MeCN)_4ClO_4$	274
Amino acids	
$Cu(II)(L\text{-tryptophanate})_2$	22,261,263,172,173,179,297
$Cu(II)(D\text{-tryptophanate})_2$	22,261,262,270,297
$Cu(II)(DL\text{-tryptophanate})_2$	22,261,262,270,297
Cu(II)(D-aspartate)	22,297
Cu(II)(L-aspartate)	22,297
$Cu(II)(L\text{-lysinate})(Cl)_2$	22,297
Cu(II)(epsilon-aminocaproate)	22,297
Cu(II)(L-tryptophanate)(L-phenylalaninate)	261,262
$Cu(II)(L\text{-phenylalaninate})_2$	261,262
$Cu(II)(glycinate)_2$	267
$Cu(II)(L\text{-asparaginate})_2$	267
Cu(II)(L-alaninate)L-(threoninate)	267
$Cu(II)(L\text{-alaninate})_2$	267
$Cu(II)(L\text{-valininate})_2$	267
$Cu(II)(L\text{-threoninate})_2$	267
$Cu(II)(L\text{-prolinate})_2$	267
$Cu(II)(L\text{-lysinate})_2Cl$	267
$Cu(II)(L\text{-lysinate})(Cl)_2$	267
Cu(II)(L-histidinate)(L-valinate)	267
Cu(II)(glycinate)(L-histidinate)Cl	267
Cu(II)(L-histidinate)(L-threoninate)	267
$Cu(II)(L\text{-glutaminate})_2$	267
$Cu(II)(L\text{-histidinate})_2$	267,268,274
Cu(II)(L-alaninadate)(L-histidinate)	267
$Cy(II)(L\text{-methioninate})_2Cl$	267
Cu(II)(L-histidinate)(L-serinate)	267
$Cu(II)(L\text{-leucinate})_2$	267
$Cu(II)(L\text{-isoleucinate})_2$	267
Cu(II)(L-cystinate)	267
$Cu(II)(L\text{-seroninate})_2$	267
Cu(II)(L-alaninatesalicylidene)	267
Cu(II)(anthranilate)(L-phenylalaninate)	267

Table 6.14. continued

Compound	*Reference*
Cu(II)(anthranilate)(L-tryptophanate)	267
NaCu(II)(L-alaninate)(salicylate)	267
Cu(II)(anthranilate)(L-methioninate)	267
Aromatic carboxylic acids	
$Cu(II)(anthranilate)_2$	22,297
$Cu(II)_2(nicotinate)_4$	22,297
$Cu(II)_2$[2-[3-(trifluoromethyl)phenyl]aminonicotinate]$_4$	22,153,297
$Cu(II)(1\text{-carboxyisoquinoline})_2$	22,297
$Cu(II)_2(2\text{-carboxyindole})_3(acetate)$	22,297
$Cu(II)(2\text{-phenyl-4-carboxyisoquinoline})_2$	22,297
Aryl acetic acids	
$Cu(II)_2$[1-(4-chlorophenyl)-2,5-dimethyl-1*H*-pyrrole-3-acetate]$_4$	153
$Cu(II)_2$[1-(*p*-chlorobenzoyl)-5-methoxy-2-methyl-3-indolylacetate]$_4$	22,297
Corticoids	
Cu(II)(17-hydroxy-3-oxo-17α-pregna-4,6-diene-21-carboxylate)$_2$	22,297
$Cu(II)_2(hydrocortisone\text{-}21\text{-}hemisuccinate)_4$	22,297
$Cu(II)_3(dexamethasone\text{-}21\text{-}phosphate)_2$	22,297
$Cu(II)_3(hydrocortisone\text{-}21\text{-}phosphate)_2$	22,297
Histamines	
$Cu(II)(histamine)(Cl)_2(HCl)_2$	22,267,297
$Cu(II)(histaminesalicylidene)(Cl)_2$	267
Penicillamines	
$Cu(I)_n$(D-penicillamine)$_n$	266,274
$Cu(II)_2$(D-pencillamine disulphide)$_2$	22,266,297
$Na_5Cu(I)_8Cu(II)_6$(D-pencillamine)$_{12}Cl$	266
Salicylates	
$Cu(II)(salicylate)_2$	22,152,263–265,274,297
$Cu(II)(3,5\text{-diisopropylsalicylate})_2$	22,272,275,297
$Cu(II)_2(acetylsalicylate)_4$	22,152,153,264,179,268, 270,272,274–277,297
Tetrazoles	
$Cu(II)_2(1\text{-phenyl-5-aminotetrazole})_2(acetate)_4$	22,297
$Cu(II)_2(1\text{-phenyl-5-aminotetrazole})_4$	22,297

(*continued*)

Table 6.14. continued

Compound	*Reference*
Miscellaneous	
Cu(II)(4-*n*-butyl-1,2-diphenyl-3,5-pyrazolidine-dione)$_2$	22,297
Cu(II)-(carnosine)	274
Cu(II)-(TRIEN)	274
Cu(II)-(Gly-His-Gly)	274
Cu(II)-(Gly-His-Gly$_2$)	274
Cu(II)-(nitrilotriacetate)	274
Cu(II)(thiourea)$_3$Cl	274
Cu(I)-(Na$_2$thiomalate)	274
Cu-Tamrabhasma	273
Cu(II)(cimetidine)$_2$	298,299
Cu(I)(cimetidine)	299

Table 6.15. ORAL ANTIULCER ACTIVITY OF NON-STEROIDAL ANTI-INFLAMMATORY AGENT COPPER COMPLEXES IN THE SHAY RAT [22]

Compound	*Antiulcer activity (μmol/kg)*	*% Copper*
Cu(II)$_2$(acetate)$_4$(H$_2$O)$_2$	564	32
Cu(II)$_2$(aspirinate)$_4$	13	15
Cu(II)(salicylate)$_2 \cdot 4$H$_2$O	11	15
Cu(II)(butazolidine)$_2$	7	9
Cu(II)$_2$(D-pen. disulphide)$_2 \cdot 3$H$_2$O	6	16
Cu(II)(3,5-DIPS)$_2$	5	13
Cu(II)$_2$(niflumate)$_4$	4	10
Cu(II)$_2$(indomethacin)$_4$	3	8

of copper in these complexes makes it clear that antiulcer activity is not dependent upon copper content. This activity is more likely to be related to the physiochemical properties of these complexes. It is also worth noting that the amount of copper in these complexes represents one-fourth to one-sixteenth of the recommended daily intake of copper (47 μmol/day), which may explain the safe and effective use of Cu(II)$_2$(aspirinate)$_4$ in the prevention and treatment of esophageal gastric ulcer in swine [272].

A subsequent comparison of the relative antiulcer activities of three different penicillamine complexes in the Shay ulcer model revealed that the water-soluble mixed-valence complex, Na$_5$Cu(I)$_8$Cu(II)$_6$(Penicillamine)$_{12}$Cl, was

Table 6.16. COMPARISON OF ORAL ANTIULCER ACTIVITIES OF PENICILLAMINE COPPER COMPLEXES, COPPER CHLORIDE AND COPPER ACETATE, IN THE SHAY RAT [266]

Compound	*ED_{50} value (μmol/kg)*		*% Cu*
	antiulcer	*antiseverity*	
$Na_5Cu(II)_6Cu(I)_8(Pen)_{12}Cl$	10	4	32
[Cu(II)penicillamine disulphide]$_2$	>56	20	18
Cu(I)(Pen)	156	57	30
$Cu(II)Cl_2(H_2O)_2$	18	35	37
$Cu(II)_2(acetate)_4(H_2O)_2$	53	55	32

Table 6.17. COMPARISON OF ORAL ANTIULCER AND ANTISEVERITY ACTIVITIES OF PROPANTHELINE BROMIDE AND AMINO-ACID AND HISTAMINE COPPER COMPLEXES IN THE SHAY RAT [267]

Compound	*ED_{50} value (μmol/kg)*	
	antiulcer	*antiseverity*
Propantheline bromide	11	11
Cu(II)(L-glycinate)$_2$	23	23
Cu(II)(L-asparaginate)$_2$	37	<15
Cu(II)(L-alaninate)(L-threoninate)	40	22
Cu(II)(L-alaninate)$_2$	87	25
Cu(II)(L-valininate)$_2$	61	44
Cu(II)(L-threoninate)$_2$	60	23
Cu(II)(D,L-tryptophanate)$_2$	61	47
Cu(II)(L-prolinate)$_2$	96	27
Cu(II)(L-lysinate)$_2$Cl	67	31
Cu(II)(L-histidinate)(L-valinate)	72	12
Cu(II)(L-glycinate)(L-histidinate)Cl	89	15
Cu(II)(L-histidinate)(L-threoninate)	89	12
Cu(II)(L-phenylalaninate)$_2$	94	27
Cu(II)(L-glutaminate)$_2$	98	17
Cu(II)(L-histidinate)$_2$	102	65
Cu(II)(L-alaninate)(L-histidinate)	114	16
Cu(II)(L-tryptophanate)$_2$	90	52
Cu(II)(D-tryptophanate)$_2$	163	–
Cu(II)(L-methioninate)$_2$Cl	101	71
Cu(II)(L-histidinate)(L-serinate)	>124	28
Cu(II)(L-leucinate)$_2$	>124	<15
Cu(II)(L-isoleucinate)$_2$	>124	19
Cu(II)(L-cystinate)	>133	46
Cu(II)(L-seroninate)$_2$	>147	18
Cu(II)(histamine)$_2$(Cl)$_2$	37	17

the most effective, and more effective than loosely bonded forms of copper, as shown in *Table 6.16* [266].

Copper complexes of amino acids have also been found to be effective in preventing and reducing the severity of Shay ulcers [267, 268]. As data in *Table 6.17* show, the copper complex of glycine was the most effective of all of the amino-acid complexes studied and it was essentially as effective as Propantheline in reducing ulcer number as well as ulcer severity [267].

The original report of antiulcer activity for copper complexes also showed that these copper complexes reduced gastric acid secretion in the Shay rat [22]. The influence of copper complexes on histamine activity was examined by studying the activity of the copper complex of histamine. Cu(II)(histamine)Cl_2 was found to be a potent *antiulcer* and *antisecretory* agent, as shown at the bottom of *Table 6.17*. This observation was confirmed and extended with reports that the mixed copper complex of tryptophan and phenylalanine, Cu(II)(Try)(Phe), inhibited histamine-, pentagastrin-, and bethanacol-induced gastric acid secretion in the Shay rat [261, 262] (*Table 6.18*). Its ED_{50} in reducing gastric acid secretion in the non-stimulated Shay rat was 77 ± 7 μmol/kg when it was administered intragastrically 1 h before ligation [262].

Table 6.18. ANTISECRETORY ACTIVITY OF Cu(II)(TRYPTOPHANATE)(PHENYLALANINATE) IN SECRETOGUE-STIMULATED SHAY RAT [261]

Compound	$\Delta\mu EqH^+/h$[a]	*% inhibition*
Pentagastrin	23.68 ± 11.23	87
Pentagastrin + Cu(II)(Try)(Phe)	2.98 ± 1.30[b]	
Histamine · 2HCl	16.54 ± 1.91	82.5
Histamine · 2HCl + Cu(II)(Try)(Phe)	2.90 ± 0.82[b]	
Bethanechol chloride	28.78 ± 5.14	82
Bethanechol chloride + Cu(II)(Try)(Phe)	5.30 ± 1.65[b]	

[a] μEqH^+ (M ± S.D.) secreted above basal value.
[b] $P < 0.01$.

These observations were confirmed and extended by Hayden, Thomas and West with reports showing that $Cu(II)_2(aspirinate)_4$ and $Cu(II)(salicylate)_2$ were more effective in the aspirin-exacerbated Shay ulcer model than either the H_1(Mepyramine) or H_2(Metiamide) histamine receptor blockers, as shown in *Table 6.19* [263]. It was also shown that $Cu(II)(salicylate)_2$ was more

Table 6.19. COMPARISON ON ORAL ANTIULCER ACTIVITY OF $Cu(II)_2$(ASPIRINATE)$_4$, Cu(II)(SALICYLATE)$_2$, AND HISTAMINE RECEPTOR BLOCKERS IN THE ASPIRIN-PRETREATED SHAY RAT [263]

Drug	*Dose (μmol/kg)*	*% inhibition*
Aspirin	278	+ 23.0 ± 8.0 (ulcerogenesis)
	555	+ 49.7 ± 8.0[a] (ulcerogenesis)
$Cu(II)_2$(aspirinate)$_4$	6	41.7 ± 5.9[b]
	12	69.5 ± 9.0[b]
Cu(II)(salicylate)$_2$	29	25.8 ± 8.3
	59	52.4 ± 8.1[b]
Mepyramine(pyrilamine)	35	10.7 ± 5.3
	88	18.1 ± 5.1
	350	21.6 ± 7.8
Metiamide	18	8.3 ± 1.7
	45	12.8 ± 2.0
	111	9.2 ± 2.5
	446	19.9 ± 5.9

[a] Significant increase ($P < 0.05$).
[b] Significant inhibition ($P < 0.05$) when compared with non-treated rats.

Table 6.20. COMPARISON OF ORAL ANTIULCER ACTIVITY OF Cu(II)(SALICYLATE)$_2$ AND HISTAMINE RECEPTOR BLOCKERS IN THE COLD-WATER STRESSED RAT [263]

Drug	*Dose (μmol/kg)*	*% inhibition*
Cu(II)(salicylate)$_2$	29	71.1 ± 6.7[a]
	59	84.6 ± 5.9[a]
Mepyramine(pyrilamine)	35	28.0 ± 6.7
	88	77.0 ± 5.0[a]
	350	79.8 ± 10.2[a]
Metiamide	18	44.6 ± 2.9[a]
	45	84.6 ± 5.2[a]
	111	87.2 ± 8.8[a]
	446	98.4 ± 7.7[a]

[a] Significant inhibition ($P < 0.05$).

effective than these histamine receptor blockers in the cold-stress ulcer model (*Table 6.20*) [263].

Copper(II)(salicylate)$_2$ was also shown to be more effective than cimetidine in preventing ulcers induced by aspirin, indomethacin or cold-stress (*Table 6.21*) [263].

Table 6.21. COMPARISON OF Cu(II)(SALICYLATE)$_2$ AND CIMETIDINE, GIVEN ORALLY, IN PROTECTING AGAINST GASTRIC LESIONS PRODUCED BY ORAL ULCEROGENIC DOSES OF ASPIRIN (2.22 mmol/kg), INDOMETHACIN (56 μmol/kg), AND COLD-STRESS IN RATS [265]

Ulcerogen	*Antiulcer agent*	*Dose (μmol/kg)*	*% inhibition (M ± S.E.)*
Aspirin	Cimetidine	148	19 ± 5
		297	16 ± 4
	Cu(II)(salicylate)$_2$	22	21 ± 6
		44	61 ± 10[a]
Indomethacin	Cimetidine	148	10 ± 3
		297	24 ± 6
	Cu(II)(salicylate)$_2$	22	37 ± 7[a]
		44	50 ± 5[a]
Cold-Stress	Cimetidine	148	38 ± 8[a]
		297	61 ± 9[a]
	Cu(II)(salicylate)$_2$	22	58 ± 8[a]
		44	79 ± 7[a]

[a] Significant at $P < 0.05$ (when compared with values obtained for non-treated rats).

Finally, Townsend and Sorenson demonstrated that Cu(II)(tryptophanate)$_2$ and Cu(II)$_2$(aspirinate)$_4$ increased the rate of healing of surgically placed glandular gastric ulcers [178]. These complexes prevented wound regression and increased the rate of re-epithelization. The appearance of replaced connective tissue components could not be distinguished from tissue of normal non-operated rats. These workers also found that treatment with these copper complexes prevented spleen, pancreas, and liver adhesions to the stomach, a constant feature associated with surgically placed glandular gastric ulcers.

Potent antiulcer activity without ulcerogenic activity [270] coupled with potent anti-inflammatory and analgesic activities distinguishes copper complexes as a unique class of antiulcer agents, since all other antiulcer drugs do not have anti-inflammatory and analgesic activities. Known copper-dependent biochemical processes can be cited to possibly account for antiulcer activities of copper complexes.

The originally reported antisecretory activity of copper complexes [22] has, at least in part, been explained as antihistaminic activity or as a modulation of the activity of histamine (*Tables 6.17–6.21*) [261, 263, 267, 269]. This suggestion is consistent with observations that copper markedly increases (50-fold) specific cimetidine bonding to brain membrane H_2 receptors [278–280]. Cimetidine has also been shown to form stable copper complexes [298, 299] and the computer-simulated interactions of it with copper in plasma have been investigated [300, 301]. It is also known that copper decreases compound 48/80 and concanavalin-A-induced releases of histamine from peritoneal mast cells [281]. Further explanation of these antisecretory effects have been attributed to modulated syntheses of prostaglandins E_2 and $F_{2\alpha}$ [271], which is consistent with other reports of copper complex modulation of prostaglandin syntheses [282–286].

Superoxide dismutase-mimetic activity [287–295] also offers an account of the reduction in number of ulcers [20, 263, 266, 268, 269], reduced severity of remaining ulcers [266, 267], and the apparent absence of wound regression in treated surgically placed gastric wounds [178]. Superoxide dismutase-mimetic activity of Cu(II)(3,5-DIPS)$_2$ was also used to account for its abolition of desoxycholate-induced colonic epithelial proliferation and to suggest an oxyradical etiology for inflammatory bowel disease [275]. These observations seem to suggest that a reduction in intestinal and gastric tissue copper-dependent superoxide dismutase (Cu-Zn SOD) and the accumulation of superoxide and other oxyradicals have an etiologic or pathogenic role in gastric and intestinal ulceration. Lipophilic copper complexes may be effective in crossing lipid cell membranes and either inducing or facilitating *de novo* synthesis of Cu-Zn SOD or disproportionating superoxide as a result of their own chemical reactivity.

Maintenance and repair of duodenal and gastric collagen and elastin connective tissue components also seems to be an important copper-dependent enzyme function in preventing or repairing duodenal or gastric ulcers. Induction or facilitation of *de novo* synthesis of lysyl oxidase by copper complexes [16] merits consideration to account for the observed rapid and normal replacement of connective tissue components in the surgically placed gastric ulcer model [178].

Recently recognized roles for copper-dependent peptidyl α-amidating enzymes in intestinal and gastric tissues for syntheses of cholecystokinin and gastrin, respectively, may also be important in understanding normal gastrointestinal physiology as well as accounting for antiulcer activity of copper complexes [12, 19].

Finally, recognizing the copper dependency of catecholamine synthesis,

dopamine-β-hydroxylase and extremely acidic copper-containing protein [6] as well as the copper dependency of amine oxidation may also contribute to a better understanding of the mechanism of action of copper complexes as antiulcer agents.

THERAPEUTIC EFFECTIVENESS OF COPPER COMPLEXES IN TREATMENT OF GASTROINTESTINAL ULCERS

Although there are historical reports of the use of copper compounds in the treatment of ulcers [138], there are as yet no modern-day uses of copper compounds for this purpose. However, since copper complexes are more effective than probanthine and cimetidine, the two most commonly used antiulcer drugs, it seems likely that copper complexes will be used for this purpose in the near future.

EPILEPSY

ALTERED COPPER METABOLISM IN CONVULSIVE DISORDERS

Epileptic patients also have elevated blood copper concentrations [302–304]. As shown in *Table 6.1*, the brain contains more copper than any other non-storage tissue in the human body [2], and brain tissues are known to require copper-dependent enzymes listed in *Table 6.2* for normal development and function [1, 8, 135]. In addition, it has been pointed out that copper-dependent processes are required for modulation of prostaglandin syntheses [135], lysosomal membrane stability [135], and the activity of histamine (see previous Section on gastrointestinal ulcers), which are also important for normal brain functions.

A symptom of copper deficiency in man and animals is seizures, which subside with copper supplementation [135, 305–310]. Seizures following treatment with tremor-inducing drugs are accompanied by a concomitant reduction in brain copper levels [311–314]. Also, brain norepinephrine and epinephrine concentrations are reduced in association with seizures [1, 311, 315–320]. This latter observation is particularly relevant, since two copper-dependent enzymes are required for the synthesis of norepinephrine and epinephrine.

The hypothesis that seizures result from the loss of copper from some copper-dependent site which may be replaced with the physiological release of liver copper stores is consistent with the above and observations that:

(1) inorganic copper injected into the carotid blood supply readily crosses the blood-brain barrier, within 15 s, and the amount crossing the barrier is increased by co-administering amino acids [213]; (2) all active antiepileptic drugs [321] can be viewed as capable of forming copper complexes; and (3) copper complexes have anticonvulsant activity (*vide infra*). Additional support for this hypothesis comes from observations that post-mortem samples of brain tissue from epileptic patients have markedly decreased copper concentrations [305] and etiologies of epilepsy in children [322] and adults [323] are associated with inflammatory conditions in the central nervous system.

ANTICONVULSANT ACTIVITIES OF COPPER COMPLEXES

Copper complexes listed in *Table 6.22* have been reported [324–327] to have anticonvulsant activity in the μmol/kg dose range following subcutaneous or intraperitoneal administration to rodent models of grand mal or petit mal seizures, maximal electroshock- or pentylenetetrazol-induced seizures [328, 329]. Some of these compounds had a rapid onset of action, within 30 min following administration, and the anticonvulsant effect persisted for up to 8 h. Others had a delayed onset of action, approximately 4 h, with a prolonged effect lasting for up to 24 h. Most of these complexes were found to be more effective in the pentylenetetrazol-induced seizure model, but lipophilic complexes were found to be effective in both models of seizure.

Initially, all compounds were given subcutaneously. Subcutaneous administration of either $CuCl_2$ or $Cu_2(acetate)_4(H_2O)_2$ failed to provide any protection against these seizures. Recognizing that ligands used to make these complexes had no anticonvulsant activity, and some were convulsants, and the lack of anticonvulsant activity following subcutaneous administration of $CuCl_2$ and $Cu_2(acetate)_4$ suggested that the observed anticonvulsant activity was due to the existence of copper complexes *in vivo*. This led to the suggestion that the active form of the antiepileptic drugs might be their copper complex formed *in vivo*. In support of this suggestion, it was found that the copper complex of amobarbital is more effective than the parent drug in protecting mice against the maximal electroshock-induced seizures [326]. In addition, this complex was free of the hypnotic (sleep-inducing) activity of the parent drug if it was absorbed slowly (subcutaneous administration) but a more potent hypnotic if absorbed rapidly (intraperitoneal administration). Even $Cu_2(acetate)_4$ had some anticonvulsant activity following intraperitoneal administration.

Copper complexes of phenobarbital, dilantin, valproic acid, lorazepam and

Table 6.22. COPPER COMPLEXES OF VARIOUS CLASSES OF COMPOUNDS REPORTED TO HAVE ANTICONVULSANT ACTIVITY [324–327]

Amino acids	Antiepileptic drugs
Cu(II)(L-threoninate)(L-serinate)	$Cu(II)(dilantin)_2$
Cu(II)(L-threoninate)(L-alaninate)	$Cu(II)_2(valproate)_4$
$Cu(II)(L\text{-valinate})_2$	$Cu(II)(phenobarbital)_2(H_2O)_3$
$Cu(II)(L\text{-threoninate})_2$	$Cu(II)(phenobarbital)_2(pyridine)_2$
$Cu(II)(L\text{-alaninate})_2$	$Cu(II)(phenobarbital)_2(imidazole)_2$
$Cu(II)(L\text{-phenylalaninate})_2$	$Cu(II)(amobarbital)_2(H_2O)_2$
$Cu(II)(L\text{-cystinate})_2$	$Cu(II)(amobarbital)_2(pyridine)_2$
$Cu(II)(L\text{-serinate})_2$	$Cu(II)(amobarbital)_2(imidazole)_2$
$Cu(II)(L\text{-tryptophanate})_2$	$Cu(II)(lorazepam)_2(Cl)_2H_2O$
$Cu(II)(L\text{-glutamate})_2$	
$Cu(II)(L\text{-leucinate})_2$	
$Cu(II)(L\text{-isoleucinate})_2$	
Cu(II)(salicylidene-L-valinate)	
Cu(II)(salicylidene-L-histidinate)	
Salicylic acids	Miscellaneous
$Cu(II)(salicylate)_2$	$Cu(II)_2(acetate)_4$
$Cu(II)(4\text{-aminosalicylate})_2$	$Cu(II)(acetylacetonethyleneimine)_2$
$Cu(II)(4\text{-tertiarybutylsalicylate})_2$	$Cu(II)(salicylatoethyleneimine)_2$
$Cu(II)(4\text{-nitrosalicylate})_2$	
$Cu(II)(3,5\text{-diisopropylsalicylate})_2$	
$Cu(II)_2(adamantylsalicylate)_4$	
$Cu(II)_2(acetylsalicylate)_4$	
$Cu(II)(acetylsalicylate)_2(pyridine)_2$	
$Cu(II)(acetylsalicylate)_2(dimethylsulphoxide)_2$	

additional complexes prepared with amobarbital were synthesized to examine the hypothesis that they might be the active form of these drugs formed *in vivo*. These copper complexes were found to be as active or, most often, more active than their parent ligands [327]. These data support the concept that copper complexes of antiepileptic drugs account for their beneficial effects in the treatment of epilepsy.

Whatever the pathological lesion causing epilepsy, it seems clear that studies of the anticonvulsant activities of copper complexes may provide a better approach to therapy of this disease. Also, a better understanding of altered essential metalloelement metabolism in epilepsy may provide a better understanding of the pathological lesion responsible for this disease.

It has been suggested that a major portion of brain copper is in the form of Cu-Zn SOD [330] and that seizures may be due to a lack of superoxide disproportionation in the brain [331]. Since copper complexes in general have

SOD-like activity, copper complexes of antiepileptic drugs were also examined for SOD-mimetic activity and found to be among the most potent of all SOD-mimetics studied while their ligands were ineffective [327]. These data suggest that copper complexes of antiepileptic drugs may have a role in facilitating the restoration of Cu-Zn SOD activity as a result of *de novo* synthesis or providing SOD-mimetic activity needed to overcome seizure states.

THERAPEUTIC EFFECTIVENESS OF COPPER COMPLEXES IN TREATMENT OF CONVULSIVE DISORDERS

There are historical uses of copper compounds for the treatment of epilepsy [138]. Unfortunately, there are no modern-day uses of copper complexes for this purpose. It is likely that copper complexes representing less than the amounts of copper recommended as safe daily intakes will eventually be found to be useful in successfully treating human seizure states.

CANCERS

ALTERED COPPER METABOLISM IN CANCERS

Copper metabolism has been studied in a variety of neoplastic diseases [332]. It is now known that patients with acute leukaemia have elevated serum or plasma copper concentrations [333]. The elevation in serum copper correlated with an increase in number of bone marrow blast cells. A decline in symptoms or remission of disease following therapy correlated with a decrease in serum copper concentration [332–335], enabling accurate prognoses based upon serum copper determinations.

Chronic leukaemia is associated with a near-normal plasma copper concentration and a markedly decreased hematocrit, which may be symptoms of copper deficiency. Since remissions do not occur in cases of chronic lymphocytic and myeloid leukaemias as well as myelomas, serum copper levels do not return to normal [332].

Children and adults with active Hodgkin's disease have elevated plasma copper concentrations. Copper levels return to normal with remission and increase with relapse, enabling accurate prognosis [336–339]. Since the return to normal copper level with remission is a constant feature of this desease, it has been suggested that a normal serum copper level be included among the criteria for complete remission [340]. Some patients in remission have been reported to have an elevated serum copper level [341]. However, it is

uncertain what stage of remission these patients had achieved or whether they were about to have a relapse. If a patient had active disease and was entering into a remission phase, it might require some time before the serum copper level returned to normal. The increase in serum copper was correlated with an increase in ceruloplasmin concentration which was attributed to a lack of catabolism by the liver [342, 343], as opposed to the alternative interpretation of increased ceruloplasmin synthesis.

A good correlation was also found between increased serum copper concentration and disease activity in non-Hodgkin's lymphomas [332]. Patients who responded to therapy had a return to normal serum copper levels, but nonresponders had a persistently elevated serum copper level [344]. Relapse was associated with an elevated copper concentration prior to the onset of symptomatic relapse.

Elevated serum copper has also been reported for patients with various carcinomas. The degree of elevation in women with cervical carcinoma increased as the stage of disease progressed and those patients who responded favourably to treatment had a nearly normal serum copper level [345]. Patients with bladder carcinoma also have elevated serum copper which was found to correlate with stages of this disease [346]. An elevation in serum copper has also been reported for patients with mammary [332, 347], bronchial [348], gastric [349, 350], colonic [350], rectal [350], and liver [350] carcinomas as well as osteosarcoma [351]. The degree of elevation in serum copper in osteosarcoma has been correlated with the extent and activity of this disease. The highest copper levels were associated with metastatic disease and the poorest prognosis [352]. Increases in serum copper with liver tumours can be partially accounted for by a failure in liver-mediated copper excretion.

Elevated serum copper levels were also found in dogs with radiation-induced and spontaneous osteosarcoma [353]. No clinical signs of metastasis were observed following tumour removal, by limb amputation, and there was a return to normal or near-normal serum copper levels. Dogs with nonmalignant nonosteosarcoma lesions were found to have normal serum copper levels. Detailed studies of altered copper metabolism in tumour-bearing mice and rats have also been recently reviewed [354].

In each of the above neoplastic diseases, the elevation of serum copper was found to correlate with disease activity and a return to normal levels was associated with remission. These observations are consistent with the view that the increase in serum copper is a physiological response which may facilitate remission.

The increase in serum or plasma copper concentration found in all cancers studied has special significance since it has recently been recognized that

many, if not all, tumour cells have decreased Cu-Zn SOD activity when compared with the Cu-Zn SOD activity in non-transformed cells [355]. The elevation of blood copper following release from liver stores may have a physiological role in activating SOD or other copper-dependent enzyme activities in cancer cells and play a role in facilitating remission and the subsequent return to normal copper levels [354]. This suggestion is supported by many observations that copper complexes have anticancer activity.

ANTICANCER ACTIVITIES OF COPPER COMPLEXES

Sharples [356] was the first to report that increasing dietary copper of 4-dimethylaminoazobenzene-fed rats lengthened hepatic tumour-induction time [357]. Inorganic copper was also found to be effective in preventing ethionine-induced liver tumours in rodents [357] and a variety of other animal carcinomas [358, 359].

However, treatment with inorganic copper was not as effective as therapy with copper complexes. A single 5 mg/kg dose of Cu(II)(dimethylglyoxime)$_2$ increased the life-span of mice bearing Ehrlich ascites or Sarcoma 180 tumours 2- to 3-times that of non-treated controls [358]. Other copper complexes reported to have similar antitumour activities in rodents are Cu(II)(3,4,7,8,-tetramethyl-1,10-phenanthroline)$_2^{2+}$ [360], Cu(II)(2-keto-3-ethoxybutyraldehyde bisthiosemicarbazone) [361–365], Cu(II)(pyruvaldehyde bisthiosemicarbazone) [366], copper complexes of 2-formylpyridine and 1-formylisoquinoline thiosemicarbozones [367, 368], copper-bleomycin [369], Cu(II)(glycylglycylhistidinate) [370], Cu(II)(glycylhistidyllysine) [371], Cu(II)(pyridine-2-carboxaldehyde-2-pyridylhydrazonate), Cu(II)(salicylaldehydebenzoylhydrazonate) [372], Cu(II) amino acids [373], Cu(II)(2,3,4-trihydroxybenzaldoximate)$_2$ [373a], and a large variety of other classes of copper complexes [374]. In addition to demonstrating antitumour activity for these copper complexes, mechanistic studies provided a great deal of information concerning their possible inhibition of DNA synthesis.

However, Oberley and Buettner pointed out that all tumour cell lines have markedly decreased superoxide dismutase activity [355]. The marked decrease in manganese-dependent SOD activity is consistent with a decrease in aerobic metabolism and a decrease or only a modest increase in Cu-Zn SOD activity is consistent with an impaired response leading to transformation. As a result of the observed lowered SOD activities in tumour cells and an awareness of the SOD-mimetic activity of copper salicylate complexes, salicylate and other copper complexes were investigated as antitumour agents.

Oberley, Leuthauser, Buettner, Sorenson, Oberley and Bize [375] initially observed that copper-dependent SOD obtained from porcine liver was effective in decreasing growth of solid sarcoma 180 tumours and prolonged the life of these tumour-bearing mice. They also found that the copper complex of aspirin, $Cu(II)_2$(acetylsalicylate)$_4$, was more effective in decreasing tumour growth and prolonging life than the porcine-derived SOD. In order to generally increase the solubility and specifically increase the lipophilicity of $Cu(II)_2$(acetylsalicylate)$_4$, which is polymeric in the solid state, both dimethylsulphoxide and pyridine monomolecular solvates were prepared. Both of these solvates were found to be more effective than the polymeric form [375]. Since an increase in lipid solubility appeared to enhance activity, an ether-soluble complex, Cu(II)(3,5-DIPS)$_2$, was also studied and found to be the most effective antitumour complex [376].

In addition to being effective against sarcoma 180 tumours, Cu(II)(3,5-DIPS)$_2$ as well as Cu(II)(salicylate)$_2$ and Cu(II)(3,5-ditertiarybutylsalicylate)$_2$ also inhibited growth of solid Ehrlich carcinomas and markedly increased the life span of mice bearing this solid tumour [375–378]. Increasing the number of treatments further increased survival time by inhibiting metastasis and decreasing tumour growth.

Treatment with Cu(II)(3,5-DIPS)$_2$ was also found to have an additive effect on survival of solid Ehrlich tumour-bearing mice when it was co-administered with the anticancer agent 1,3-bis(2-chloroethyl-2-nitrosourea) (BCNU). A single dose of 14.5 mg/kg Cu(II)(3,5-DIPS)$_2$ was essentially ineffective in increasing survival, and a 30 mg/kg dose of BCNU produced only 15% survival while the combination of these two agents produced 60% survival through the course of the experiment [378]. These results were interpreted as being consistent with the SOD-mimetic activity of Cu(II)(3,5-DIPS)$_4$.

SOD-mimetic activity of Cu(II)(3,5-DIPS)$_2$ also led to studies of this compound's ability to inhibit interleukin-2 (IL-2) synthesis by a mouse thymoma cell line, EL4 [379]. Cu(II)(3,5-DIPS)$_2$ did inhibit phorbol diester-induced synthesis of IL-2 (IC_{50} = 10 μM), but it did not inhibit phorbol-diester-induced attachment of these cells to substrate. While 3,5-DIPS also inhibited IL-2 synthesis (IC_{50} value = 15 μM), $CuCl_2$ was ineffective (100 μM). Mechanistically, the inhibition of IL-2 synthesis by Cu(II)(3,5-DIPS)$_2$ was suggested to be due to an inhibition of IL-2 messenger RNA transcription [379].

Leuthauser also observed that solid Ehrlich tumours taken from mice treated with Cu(II)(3,5-DIPS)$_2$ contained differentiated epithelial cells in duct arrangement, suggesting that Cu(II)(3,5-DIPS)$_2$ treatment did not kill these tumour cells but caused them to differentiate to normal duct cells [380]. This observation was confirmed and extended by Sahu in his dissertation [381]. He

found that Cu(II)(3,5-DIPS)$_2$ added to neuroblastoma culture medium caused differentiation of these neoplastic cells to normal neuronal cells in a concentration-related manner. If Cu(II)(3,5-DIPS)$_2$ does not kill transformed cells but instead causes them to differentiate to normal cells, the future use of copper complexes to treat neoplastic diseases has some exciting possibilities.

THERAPEUTIC EFFECTIVENESS OF COPPER COMPLEXES IN TREATMENT OF CANCERS

There is a report of complete osteosarcoma regression in a single patient treated with vitamin C and Cu(II)(glycylglycylhistidinate) [382]. X-Ray evidence of osteosarcoma before treatment and its absence after treatment supports this claim. The above anticancer activities as well as the anticarcinogenic and antimutagenic activities of copper complexes (see next section) support the conclusion that they merit further study as potentially useful compounds for the treatment of human cancers. Wattenberg has suggested that Cu(II)(3,5-DIPS)$_2$ is representative of a class of SOD mimetics which have potential as chemopreventive agents [383].

CARCINOGENESIS

ALTERED COPPER METABOLISM IN CARCINOGENESIS

Carruthers and Suntzeff [384] were the first to show that copper metabolism was altered in tissue undergoing carcinogenesis. They demonstrated that applying a benezene solution of a carcinogen, methycholanthrene, to mouse skin initially caused hyperplasia which was associated with a 45% reduction in tissue copper content. When application of the carcinogen was continued beyond the initiation stage, carcinomas developed and these tumours had a copper content of only 15% of normal at the end of the promotion stage. The frequent application of a sufficiently high concentration of carcinogen, sufficient to initiate and promote tumour development, was the early model of carcinogenesis.

Subsequent developments led to the observation that the application of a less concentrated solution, which was high enough to initiate hyperplasia but too low to promote tumour development, could be used to produce tumours if its application was followed by the application of an irritant, such as a phorbol diester, capable of promoting neoplastic tumour development. The

initiation of carcinogenesis with the lower concentration of carcinogen followed by promotion of carcinogenesis with a phorbol diester has also been shown to affect copper metabolism. This procedure was found to cause a 45% decrease in Cu-Zn SOD activity [385, 386] and it is likely that the 85% reduction in tumour copper found by Carruthers and Suntzeff [384] was in large part due to a reduction in Cu-Zn SOD.

These observations of altered copper metabolism in carcinogenesis are particularly important, since exogenously administered copper complexes have anticarcinogenic activity. This suggests that endogenous copper complexes may have a physiological role in responding to hyperplasia and prevention of carcinogenesis [354] and exogenous copper complexes represent a novel class of cancer chemoprotective agents [386].

ANTICARCINOGENIC ACTIVITIES OF COPPER COMPLEXES

Kensler, Bush and Kozumbo were the first to show that Cu(II)(3,5-DIPS)$_2$ inhibited 7,12-dimethylbenz[*a*]anthracene-(DMBA)-initiated 13-acetyl-12-tetradecanoylphorbol-promoted tumour development in the skin of mice [387]. A 2.0 μM diethyl ether solution of Cu(II)(3,5-DIPS)$_2$ applied onto the skin of these mice caused a 60% inhibition of tumour incidence and a 93% reduction in number of tumours per mouse. A 2 μM solution of Cu(II)(3,5-DIPS)$_2$ was also found to inhibit carcinogenesis when DMBA was used as a complete carcinogen, without the use of the phorbol diester as a promoter [386–390]. Since Cu(II)(3,5-DIPS)$_2$ inhibits the covalent bonding of DMBA to epidermal DNA [391], these anticarcinogenic effects may be mediated through modulation of cytochrome *P*-450 reductase [392] or cytochrome *P*-450 [288]. No significant inhibition of tumour formation was observed with Cu_2(acetate)$_4$ or 3,5-DIPS acid [388]. The notion that it is the copper complex that is required for anticarcinogenic activity was further supported by results of Kensler and Trush, demonstrating that Cu(II)(3,5-DIPS)$_2$ was an effective inhibitor of chemiluminescence due to phorbol diester-promoted release of superoxide from phagocytic cells challenged with this diester, where neither Zn(II)(3,5-DIPS)$_2$ nor 3,5-DIPS acid had any inhibitory activity [389]. Kensler and Trush also found that Zn(II)(3,5-DIPS)$_2$ and 3,5-DIPS acid had no SOD-like activity in four different test systems, while Cu(II)(3,5-DIPS)$_2$ caused a 50% inhibition of superoxide-mediated reactions at concentrations ranging from 0.2 to 3.2 μM [389]. These data illustrate an obligatory role of the copper complex in mediating this anticarcinogenesis.

Induction of ornithine decarboxylase is another feature of epidermal exposure to the phorbol diester. Induction of this enzyme is also inhibited by

Cu(II)(3,5-DIPS)$_2$ [386]. Inhibition was found to be directly related to the applied concentration and the concentration producing 50% inhibition was 1.0 μM. Cu$_2$(acetate)$_4$, Cu(II)(EDTA), CuSO$_4$, Zn(II)(3,5-DIPS)$_2$, and 3,5-DIPS acid were ineffective at concentrations ranging from 5 to 20 μM [387, 389, 390]. Most recently [386], Cu(II)-(hydrocortisone) and Cu(II)$_2$(indomethacin)$_4$ complexes were also reported to inhibit ornithine decarboxylase *in vivo*, and this inhibitory activity exceeded the activities of their parent ligands.

The finding that lipophilic copper chelates with SOD-like activity can inhibit phorbol diester-induced biochemical and biological responses, including neoplastic transformation [386a], strengthens arguments for a role of oxygen radicals in carcinogenesis, perhaps more so in stage II rather than stage I promotion [386, 393], and a role for Cu-SOD or SOD-like compounds in prevention of carcinogenesis as chemoprotective agents under physiological conditions.

ANTIMUTAGENIC ACTIVITIES OF COPPER COMPLEXES

In addition to confirming Kensler's observation that Cu(II)(3,5-DIPS)$_2$ has anticarcinogenic activity, Solanki, Yotti, Logani and Slaga [394] demonstrated that this complex has antimutagenic activity also. DMBA-induced mouse keratinocyte-mediated Chinese hamster V-79 cell mutagenesis was inhibited and replating efficiency increased in a dose-related manner. Concentrations ranging from 0.01 to 4 μg of Cu(II)(3,5-DIPS)$_2$ per ml of culture medium produced up to 60% inhibition of mutagenesis and increased challenged replated-cell viability from 64 to 100%.

Using the Ames *Salmonella typhimurium* revertant assay to study the mechanism of action of Cu(II)(3,5-DIPS)$_2$, Reiners, Brott and Sorenson [395] found that Aroclor-1254-stimulated SENCAR mouse liver cells treated with varying concentrations of Cu(II)(3,5-DIPS)$_2$ resulted in a dose-related noncompetitive inhibition of benzo[*a*]pyrene mutagenesis. Cytochrome *P*-450 or the cytochrome *P*-450 reductase activity of liver cell homogenates and microsomal preparations were inhibited by antimutagenic concentrations of Cu(II)(3,5-DIPS)$_2$. Neither 3,5-DIPS nor Zn(II)(3,5-DIPS)$_2$ inhibited mutagenesis or cytochrome *P*-450 activation of benzo[*a*]pyrene. CuSO$_4$, while less active than Cu(II)(3,5-DIPS)$_2$, did inhibit mutagenesis and *P*-450 activation, which may be indirectly due to the formation of some copper complex or complexes in this system. The antimutagenic activity of Cu(II)(3,5-DIPS)$_2$ and CuSO$_4$ was not attributed to their SOD-mimetic activity, since bovine Cu-Zn SOD, at a 100-fold excess of Cu(II)(3,5-DIPS)$_2$ SOD-mimetic activity,

had no effect on their antimutagenic activity. However, this rationale is open to further examination, since Cu-Zn SOD may be too large to fit into or near the site of superoxide production, the oxyferricytochrome intermediate, whereas low-molecular-weight complexes may be easily accommodated at this site.

THERAPEUTIC EFFECTIVENESS OF COPPER COMPLEXES IN TREATMENT OF CARCINOGENESIS

The anticancer, anticarcinogenic, and antimutagenic activities of copper complexes do support suggestions that SOD-mimetic complexes such as $Cu(II)(3,5\text{-}DIPS)_2$ may be useful in chemoprevention of cancers and carcinogenesis [383, 396] in man and, as a result, merit in-depth study [397].

DIABETES

ALTERED COPPER METABOLISM IN DIABETES

Impaired glucose tolerance in animals associated with copper deficiency and its correction by copper supplementation was originally reported by Keil and Nelson in 1934 [398]. This observation has been confirmed [399, 400] and extended with reports that dietary carbohydrate can exacerbate (fructose > glucose > sucrose) this copper deficiency-induced diabetogenic state associated with increased blood glucose, glucosuria and decreased insulin secretion [400]. Feeding these reducing sugars exacerbated many symptoms of copper deficiency, including decreases in plasma ceruloplasmin, liver copper content, plasma insulin, glucose utilization and tolerance, insulin receptor bonding, lipogenesis and epidimal fat, blood haemoglobin and hematocrit, growth, intestinal hexose uptake, liver and diaphragm glycogen, serum albumin, systolic blood pressure, erythrocyte Cu-Zn SOD, hepatic ATP and glucose-6-phosphatase activity as well as increases in uptake of ^{67}Cu with increased retention of ^{67}Cu in the gastrointestinal tract and impaired absorption, streptozotocin diabetes, hepatic weight, iron content, blood urea nitrogen, ammonia, triacylglycerols and cholesterol, hepatic glucose-6-phosphate dehydrogenase, malic enzyme, L-α-glycerophosphate dehydrogenase, and fructose-1,6-bisphosphatase activities, heart size (hypertrophy with histopathologic changes), and mortality [401–417]. The reducing sugar content of human diets is also known to affect glucose tolerance. Ingestion of a high-sugar diet composed of mono- and disaccharides significantly ($P < 0.05$) lowered insulin-receptor bonding [414].

Humans fed a typical American diet containing fructose and low copper led to the elimination of 4 of the original 24 individuals who exhibited heart-related abnormalities and reduced erythrocyte Cu-Zn SOD [418]. Repletion of the remaining subjects' diet with copper significantly increased Cu-Zn SOD levels and demonstrated that this dietary carbohydrate does affect indices of copper status in humans. Decreased glucose tolerance was observed in two men during experimental copper depletion, 0.78 mg daily, but improved with repletion, 6 mg of copper daily, beyond the glucose tolerance observed before the initiation of depletion [419]. Glucose tolerance was suggested to be a better indicator of copper nutriture than changes in haematology, plasma copper, ceruloplasmin, cholesterol or erythrocyte Cu-Zn SOD. These results were also suggested to be important with regard to the etiology or pathophysiology of mild diabetes which always precedes more severe diabetes. These observations are relevant to patients with Type 1 diabetes mellitus, since they do have decreased striated muscle copper content and their significantly ($P < 0.05$) elevated plasma copper levels do decrease with metabolic control of their diabetes [420].

Additional diabetes related studies in rats or mice fed copper-deficient diets document impaired synthesis or release of thyroid stimulating hormone in response to thyrotropin-releasing hormone [421], atrophy of pancreatic acinar tissue [422, 423, 424] impaired release of pancreatic amylase [413], and increased glycosylated haemoglobin [425].

Streptozotocin and alloxan are well-known diabetogenic agents which produce hyperglycaemia, glucosuria, increased food and water consumption and depressed growth in rats and mice [426]. Injection of streptozotocin is known to cause a time-related increase in renal cortex levels of metallothionein-bonded copper [427–430]. This increase has been suggested to be due to increased absorption [430] resulting in nephropathy associated with chronic diabetes, however, there was no evidence for either an increase in absorption or nephropathy in these animals and the possibility that this accumulation of copper in the kidney was due to a copper conservation role of the kidney was not excluded.

Impaired glucose utilization in rats treated with streptozotocin was corrected in rats fed a copper-supplemented diet [400] and copper added to *in vitro* preparations of diaphragm muscle and epididymal fat cells stimulated glucose utilization, increasing diaphragm glycogen deposition and epididymal lipid [400]. These observations were consistent with improved glucose tolerance and increased epididymal lipogenesis when streptozotocin-diabetic rats were treated with copper and insulin [416]. Adipocytes from normal rats treated with copper evidenced a 3-fold increase in insulin bonding and increased

glucose transport, suggesting that copper stimulated lipogenesis and glycogenesis [417, 431].

Recent animal studies have documented that the diabetogenic agents, streptozotocin and alloxan, inhibit pancreatic islet β-cell Cu-Zn SOD and decrease glucose-induced insulin secretion [432–439]. These observations led to reports that treatment with Cu-Zn SOD inhibited streptozotocin- or alloxan-induced diabetes [435, 436, 438, 440], reduce hyperglycaemia and decreased pancreatic glucagon; however, the derivatized enzyme did not affect blood glucose levels [441, 442] or protect islet cells against streptozotocin impaired glucose-stimulated insulin release [442]. A single injection of alloxan kills insulin-producing pancreatic islet β cells and causes diabetes mellitus in animals as a result of the production of superoxide, which may also produce islet inflammation [437], which often accompanies the onset of human diabetes [443]. Human pancreatic islet β cells are rich in Cu-Zn SOD [432, 434] and a decrease in the concentration or activity of this enzyme may be relevant to β-cell damage and the pathogenesis of insulin-requiring diabetes [432, 434].

These results support the hypothesis that superoxide or other oxygen radicals derived from it such as singlet oxygen [444] and hydroperoxyl or hydroxyl radicals may mediate β-cell damage and the decrease in insulin, resulting in insulin-dependent diabetes [435].

ANTIDIABETIC ACTIVITY OF COPPER COMPLEXES

Following the report that Cu-Zn SOD had antidiabetic activity in the streptozotocin-diabetic rat [435], SOD mimetic copper complexes were studied and found to have antidiabetic activity in this model of diabetes mellitus [445, 446]. Impaired glucose utilization in streptozotocin-treated mice was not corrected by doses of 0.23 mmol/kg salicylic acid or up to 0.18 mmol/kg 3,5-DIPS when compared to the control (vehicle treated) group at time zero. With injection of glucose there was a more marked impairment of glucose utilization as evidenced by the subsequent rise in plasma glucose over the 60 min glucose tolerance test period. Treatment with 0.11 mmol/kg Cu(II)(salicylate)$_2$ or doses of 0.02, 0.09 or 0.24 mmol/kg Cu(II)(3,5-DIPS)$_2$ did produce an antidiabetic effect, as evidenced by lower plasma glucose values at time zero of the glucose tolerance test and glucose tolerance was significantly ($P < 0.001$) improved and related to dose in three Cu(II)(3,5-DIPS)$_2$-treated groups when compared with the streptozotocin-treated group. Plasmaglucose levels at 15 and 30 min after Cu(II)(salicylate)$_2$ and Cu(II)(3,5-DIPS)$_2$ treatment were normal, eliminating the possibility of protection via hyperglycaemia at the time of streptozotocin injection, and liver function tests were normal at the

time of glucose tolerance testing. A slight elevation of blood urea nitrogen was observed in rats receiving Cu(II)(3,5-DIPS)$_2$ (22–28 mg/100 ml vs. 13–20 mg/100 ml in controls). Glucosuria for rats treated with streptozotocin alone or streptozotocin plus 3,5-DIPS was 3–4+ and 2–3+ for streptozotocin plus salicylic acid. Glucosuria for Cu(II)(3,5-DIPS)$_2$-pretreated rats was 0–1+ at all doses studied and 0–2+ in the Cu(II)(salicylate)$_2$-treated group. Thus, hepatic dysfunction or glucosuria did not account for the improved glucose tolerance.

These data suggest that Cu-Zn SOD-mimetic copper complexes have chemical reactivity that can attenuate streptozotocin diabetes. This activity is attributed to the combination of superoxide disproportionating reactivity and lipophilicity of these complexes, which may allow cellular penetration and re-establishment of disproportionating reactivity or a facilitation of *de novo* synthesis of pancreatic β-cell Cu-Zn SOD. This suggestion has merit, since it has been demonstrated that streptozotocin decreases Cu-Zn SOD activity in pancreatic β-cells [433] and intravenous administration of Cu-Zn SOD attenuates streptozotocin diabetes [435, 438, 440]. Testing Cu-Zn SOD-mimetic copper complexes for their ability to prevent or attenuate experimental diabetes may be worthwhile, since it is possible that the etiology of Type 1 diabetes involves oxygen-radical-mediated β-cell damage following the loss or reduction of Cu-Zn-SOD activity. Other mechanistic possibilities relating to pancreatic islet β-cell function, insulin synthesis, insulin-receptor bonding and glucose utilization also merit consideration.

The hypoglycaemic activity and decrease in glucosuria of these salicylate complexes provide some additional understanding of the 75-year-old observation that preceded the advent of insulin, that high doses of salicylic acid (4 mmol/kg) have hypoglycaemic activity and decrease urinary excretion of glucose [447]. While a much higher dose of salicylic acid is required to reduce blood glucose and decrease glucosuria, the fact that copper chelates of salicylates are effective at much lower doses supports the notion that the earlier observed antidiabetic activities of salicylates may have been due to the formation of their copper chelates *in vivo*.

THERAPEUTIC EFFECTIVENESS OF COPPER COMPLEXES IN TREATMENT OF DIABETES

There are no current or recognized historical reports of the use of copper chelates as antidiabetic agents. However, the effectiveness of salicylates in decreasing glucosuria and hyperglycaemia in both maturity-onset and juvenile

types of diabetes [447] coupled with the antidiabetic activities of copper salicylates strongly supports their consideration in a physiological approach to the treatment of diabetes.

RADIATION INJURY

ALTERED COPPER METABOLISM IN RADIATION INJURY

Acute radiation syndromes following total-body irradiation have been described in detail [448–450]. The hematopoietic syndrome produced with radiation doses ranging from 2 to 10 Gy (100 rads = 1 Gy) for most mammals, causes bone marrow aplasia, leukopoenia, thrombocytopoenia, and anaemia as evidenced by infection and haemorrhage, which are the principal causes of death within 1–6 weeks. The gastrointestinal syndrome produced with doses ranging from 7 to 50 Gy causes epithelial mitotic arrest, denudation, and ulceration of the small bowel as evidenced by fever, diarrhoea, and disturbances in fluid and electrolyte balances, which are the primary factors leading to death within 3–5 days post-irradiation. This syndrome includes pathological changes and symptoms associated with concomitant bone marrow depression. The central nervous system or cerebrovascular syndrome results with doses ranging from 50 to 100 Gy, which cause inflammatory changes in the cerebral blood vessels, meninges and brain, as well as oedema and pyknotic changes in cerebellar granule cells and are evidenced by apathy and drowsiness followed by tremors, convulsions and ataxia prior to death, which occurs within 2 days. This syndrome includes components of the haematopoietic and gastrointestinal syndromes, but consequences of these are trivial in comparison to the total loss of brain function. Until recently, etiologies of these syndromes were less well understood with regard to chemical consequences of ionizing radiation.

It is now understood that oxygenated aqueous solutions exposed to high energy gamma- or X-rays yield radiolytic products according to the following reaction:

$$h\nu + H_2O \rightsquigarrow e^- + H^\bullet + H_3O^+ + HO^\bullet + H_2O_2.$$

The energy-rich radicals $H^\bullet$ and e^- have been suggested [451–454] to lead to superoxide formation in the presence of dioxygen according to the following reactions:

$$H^\bullet + O_2 \rightarrow HO_2^\bullet \rightarrow H^+ + O_2^{-\bullet} \text{ and } e^- + O_2 \rightarrow O_2^{-\bullet}$$

Since the copper-dependent and zinc modulated superoxide dismutase (Cu-Zn SOD) is normally found in cytosol of cells of all aerobic organisms

and has a suggested protective role in scavenging superoxide:
$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$ [455]
at a rate, $k = 1.3 \times 10^9\ M^{-1}\ s^{-1}$ [456], which is essentially diffusion-controlled, Petkau, Chelack, Pleskach, Meeker, Brady, Kelley and Barefoot studied its effectiveness as a radioprotectant in mice. Intravenous administration of the enzyme was found to increase radioresistance of treated mice [457–461]. These results are consistent with what is suggested as the normal biochemical role of Cu-Zn SOD and have been interpreted as explaining the well known 'oxygen-effect' of ionizing radiation, the increase in radiation sensitivity associated with the presence of oxygen and the decrease in radiation sensitivity in the absence of oxygen [462, 463]. Radioprotective effects of Cu-Zn SOD have also been demonstrated in cell culture systems. Diethyldithiocarbamate, a chelating agent which inhibits Cu-Zn SOD [464, 465], decreases radioresistance as well as enhancing the hyperthermic radiosensitization of Chinese hamster cells [466–469]. In addition, when Chinese hamster cells were mixed with mature erythrocytes (RBC), which contain a large concentration of Cu-Zn SOD, and then exposed to the xanthine oxidase superoxide-generating system they were totally protected from effects of generated superoxide [470–471]. This is consistent with the hypothesis that superoxide was disproportionated by RBC Cu-Zn SOD after passing through the RBC membrane. Adding RBC to cultures of human lymphoid cells also protected them from ionizing radiation-induced chromosomal damage [470, 471]. Adding Cu-Zn SOD to cultures of myoblasts protected them from a radiation-induced decrease in viability and altered morphology [472] as well as chromosomal damage [473]. Bacteria [462, 463], lymphocytes [474] and mycoplasma [475] have also been protected from ionizing radiation when Cu-Zn SOD is added to culture media. Following the report that radioprotection of mice with Cu-Zn SOD also afforded protection of RBC in these animals [461], it was demonstrated that mature human and porcine RBC were also protected with Cu-Zn SOD [466–468, 476–480]. All of these observations are consistent with the recognition that intravenously administered Cu-Zn SOD, which is too large to cross the RBC membrane, afforded protection by reducing the concentration of extramembranous superoxide.

Ionizing radiation has also been studied with regard to its effect on tissues from whole-body irradiated mice, rabbits and dogs following the suggestion that Cu–Zn SOD might be a naturally occurring radioprotective agent and that its content in a given tissue might be directly related to tissue radioresistance [481–487]. Pursual of reports of these studies leads to the conclusion that the relative radiosensitivity of tissues can be correlated with tissue Cu-Zn SOD content and tissue cell mitotic rate. Post-irradiation hematopoietic

repair accompanied by an increased number of reticuloytes in circulating blood was also suggested to account for radioresistance of survivors [488]. In general, post-irradiation increases in Cu-Zn SOD activity occurred in tissues which were more radioresistant and less mitotically active, while low activities were found for the more radiosensitive and most mitotically active tissues. Systematic studies are needed to explain post-irradiation changes in tissue Cu-Zn SOD activities.

One possible explanation for the apparent increase in Cu-Zn SOD activity of the spleen, RBC and surviving bone marrow cells, and the apparent lack of radiosensitivity of the heart, skeletal muscle and brain, as well as the slight but not significant decrease in liver Cu-Zn SOD activity may be related to normal serum copper-containing components, which increase as an acute phase response following all types of tissue irritation or damage [488]. This increase in serum copper-containing components is due to increased synthesis, in the liver, of ceruloplasmin, which contains 6 to 8 atoms of copper, and its secretion into the blood along with amino-acid copper complexes which are normal constituents of serum or plasma [5]. Increased liver synthesis and secretion of ceruloplasmin and copper amino-acid complexes into blood is a well-known acute phase response to many stimuli leading to a 2- to 3-fold increase in concentration of these normal blood plasma components [30]. Ceruloplasmin and amino-acid copper complexes are known to have SOD-like activity [287–295, 489]. Amino-acid copper complexes disproportionate superoxide at rates ranging from 0.5 to 3.0×10^9 M^{-1} s^{-1} [456] which approach and exceed the rate of Cu-Zn SOD disproportionating reactivity, 1.3×10^9 M^{-1} s^{-1}. Ceruloplasmin, which has a molecular weight of about 130,000 and is present in interstitial fluid, may protect against extracellular superoxide accumulation, and amino-acid copper complexes, which can penetrate all cell membranes because they are lipophilic, could protect against intracellular superoxide accumulation or facilitate *de novo* synthesis of Cu-Zn SOD. Thus, radioresistance of various tissues may be partially accounted for as a result of mobilization of liver copper in the form of plasma copper-containing components in response to irradiation-induced tissue damage. The liver also serves as the storage organ for copper, which is stored as the copper metallothioneine complex, copper-thioneine. This form of stored copper is also likely to have SOD-like activity and perhaps, in part, account for radioresistance of the liver. This speculation has recently been substantiated in the report that rabbit liver metallothioneine doses scavenge hydroxyl and superoxide radicals generated by the xanthine-xanthine oxidase system at high rates, 10^{12} M^{-1} s^{-1} and 10^6 M^{-1} s^{-1}, respectively [490]. In addition, copper is known to have a role in maintaining normal RBC membrane characteristics,

since treating copper-deficient animals with copper supplements restores normal RBC permeability and osmotic fragility [491]. Studies of changes in plasma and tissue copper contents in response to irradiation may contribute to a better understanding of the relative radioresistances of various tissues.

Data have been provided to support the suggestion that RBC Cu-Zn SOD activity might be used as an index of Cu-Zn SOD activity in other tissues [491–495]. Such an index has been suggested to be useful in detecting individuals who may be more radiosensitive and at greater risk with regard to acute and chronic radiation exposure [492]. Studies of changes in tissue copper content in response to irradiation may also provide a better measure of risk to normal tissues of individuals who are to undergo radiotherapy. Treatment with a copper complex does reduce risks of exposure to ionizing radiation.

It has been understood for many years that exposure to ionizing radiation increases the incidence of neoplasia in various tissues. Such exposures induce malignant transformations in mouse 10T1/2 cells in a dose-related fashion [496]. X-Ray induced transformations can be enhanced by post-irradiation incubation with a phorbol diester tumour promoter.

When it was recognized that the clastogenic effect of this phorbol diester involved the release of superoxide, clastogenicity was suppressed and cell survival increased by concomitant incubation with Cu-Zn SOD [497–499]. Pretreatment with Cu-Zn SOD also reduced X-ray and phorbol diester promoted transformations and increased survival of culture hamster embryo cells [493, 500]. In still another study it was found that Cu-Zn SOD reduced transformation of 10T1/2 cells produced by concomitant X-ray irradiation and exposure to misonidazole, a hypoxic cell radiosensitizer [501]. These observations were most pronounced when Cu-Zn SOD was present during fixation and expression periods [502], consistent with the observation that Cu-Zn SOD protects DNA [503] and proteins [474] against ionizing radiation. Effective radiation protection as a result of superoxide removal does offer anticlastogenic activity as well.

If Cu-Zn SOD has radioprotectant activity because of its ability to disproportionate superoxide [504], then small molecular weight copper complexes, which are also known to disproportionate superoxide [287–295], might also have radioprotectant activity. Recent studies document radioprotectant activity for Cu(II)(3,5-DIPS)$_2$ which has superoxide disproportionating reactivity.

RADIATION PROTECTIVE EFFECTS OF COPPER COMPLEXES

The Cu-Zn-SOD-mimetic activity of Cu(II)(3,5-DIPS)$_2$ and its lipid solubility were criteria used to select this complex in the initial attempt to study a copper complex as a radioprotectant. This complex was found to prevent death in 58% of the lethally irradiated (10 Gy, 0.4 Gy/min) mice when they were thought to have been treated with 0.49 mmol/kg, 3 h before irradiation [505]. Subsequent studies revealed that this protection could be achieved with a much lower dose of complex suspended in a propyleneglycol-poly(vinyl alcohol)-saline vehicle. Treatment with 0.16 mmol/kg produced a 60% increase in survival in similarly irradiated and treated mice [506]. Most recent studies suggest that this radioprotectant effect may be achieved with a still further reduced dose of 0.04 mmol/kg and this lower dose appears to be as effective if given 3 h after irradiation as it is when given 3 h before irradiation.

Radiation protection was also found when the radiation-dose rate was increased to 1.6 Gy/min. A dose of 0.16 mmol/kg Cu(II)(3,5-DIPS)$_2$ produced increases in survival of 7%, 46%, 36% or 20% greater than survival of vehicle-treated mice when both Cu(II)(3,5-DIPS)$_2$-treated and vehicle-treated groups were irradiated at 7, 8, 9 or 10 Gy, respectively. Ongoing studies continue to clearly show that these increases in survival of Cu(II)(3,5-DIPS)$_2$-treated mice are due to rapid recovery of immunocompetency.

Increased survival of irradiated Cu(II)(3,5-DIPS)$_2$-treated mice compared with vehicle-treated mice was related to stimulation of spleen and bone marrow hematopoiesis [507–509]. Treatment with Cu(II)(3,5-DIPS)$_2$ accelerated the recovery of spleen and bone marrow cells by stimulating hemopoietic activity in these radiation-damaged tissues, as measured by both *in vitro* and *in vivo* assays. The number of splenic mixed multi-potential colony-forming cells in Cu(II)(3,5-DIPS)$_2$-treated mice was increased to as much as 540% of the number of these cells in non-irradiated control mice. Splenic granulocyte-macrophage colony forming cells were also significantly increased in Cu(II)(3,5-DIPS)$_2$-treated mice. The recovery of bone marrow hematopoiesis, as judged by colony-forming cells assay, was also enhanced by Cu(II)(3,5-DIPS)$_2$ treatment but was delayed in comparison with splenic hematopoiesis. Cu(II)(3,5-DIPS)$_2$ stimulation of hemopoietic activity also led to accelerated recovery of T- and B-lymphocyte mitogen and antigen responsiveness following irradiation. Most recently, it has been found that Cu(II)(3,5-DIPS)$_2$ treatment produces a marked increase in immunostimulation in non-irradiated normal mice [510].

THERAPEUTIC EFFECTIVENESS OF COPPER COMPLEXES IN PREVENTION OF RADIATION INJURY

Use of copper complexes as adjunct radioprotectant treatment for prevention of radiation damage of normal tissues in individuals undergoing radiotherapy of neoplastic disease has special merit. It is well known that plasma copper concentrations increase in response to many neoplastic diseases and decrease when remission occurs [84]. The increase in plasma copper has been interpreted as a physiological response, which facilitates remission, in the light of observations that copper complexes have anticancer activity [511] and, in particular, Cu(II)(3,5-DIPS)$_2$ has anticancer activity [376–378, 512], anticarcinogenic activity against phorbol-diester-promoted tumour development (anticlastogenic activity) [387–390, 394, 512, 513], and antimutagenic activity [394]. Distribution of Cu(II)(3,5-DIPS)$_2$ to tumour tissue, as a result of its use as a radioprotectant in patients with neoplastic disease who undergo radiation therapy, may also produce an antitumour effect, rather than protecting the tumour tissue, in addition to protecting normal tissue from the effects of ionizing radiation. The utility of Cu(II)(3,5-DIPS)$_2$ and other SOD-mimetic complexes in chemoprevention or suppression of cancers in man has recently been suggested based upon its antitumour, anticarcinogenic and antimutagenic activities [383, 396].

In addition, the documented radioprotectant activity of Cu(II)(3,5-DIPS)$_2$ [505] is consistent with its anti-inflammatory activity [22, 84, 514], which relates to protection against the hematopoietic syndrome, its antiulcer activity [22, 84, 91, 514–516], which relates to its potential ability to protect against the gastrointestinal syndrome, and its anticonvulsant activity [324, 326, 516], which relates to its potential to protect against the central nervous system syndrome.

Evidence has been provided that the anti-inflammatory, anticancer, and anticarcinogenic effects of Cu(II)(3,5-DIPS)$_2$ are related to its Cu-Zn-SOD-mimetic activity [375–378, 510, 512–515]. Cu-Zn-SOD-mimetic activity may also, in time, be shown to, at least in part, account for the antiulcer, antimicrobial, and anticonvulsant effects of copper complexes. Acute and especially chronic diseases are being investigated worldwide with regard to the plausible role of superoxide in their etiologies.

The radioprotectant activity of Cu(II)(3,5-DIPS)$_2$ may not be solely due to its disproportionation of superoxide, $k = (1–2) \times 10^9\ M^{-1}\ s^{-1}$ [517], but may also be related to its reactivity with hydroxyl radical, hydroperoxyl radical, hydrated electrons, and hydrogen atoms according to the following reactions at biological pH values [324, 462, 518–520]:

$$Cu(II)(L)_2 + HO^{\bullet} \xrightarrow{(0.2-5) \times 10^9\ M^{-1}\ s^{-1}} Cu(III)(L)_2 + HO^-$$

$$H^+ + HOO^{\bullet} \xrightarrow{(1-8) \times 10^9\ M^{-1}\ s^{-1}} H_2O_2 + O_2$$

$$Cu(II)(L)_2 + e^-_{aq} \xrightarrow{(0.3-30) \times 10^9\ M^{-1}\ s^{-1}} Cu(I)L + L^{-1}$$

$$Cu(II)(L)_2 + H^{\bullet} \xrightarrow{(0.06-0.6) \times 10^9\ M^{-1}\ s^{-1}} Cu(I)L + HL$$

These reaction rates show that these reactions are very rapid and limited only by the rate of diffusion of reactants. While it is uncertain as to whether or not these very reactive products of ionizing radiation would reach a copper complex before reacting with some other cellular component or dioxygen, if they did collide and react with a copper complex some protection would result.

In support of the above pharmacological effects, Cu(II)(3,5-DIPS)$_2$ may contribute to other important copper-dependent physiological and biochemical responses required to overcome the effects of ionizing radiation. Symptoms of hematopoietic syndrome, including bone marrow aplasia, leukopoenia, anaemia and infection are also well-known symptoms of copper insufficiency [511, 521]. The turnover of ^{64}Cu in plasma and its uptake by bone marrow are greatest in anaemic and polycythemic rats because of the need for copper in bone marrow-hematopoiesis [522]. Copper-containing plasma components, ceruloplasmin as well as small-molecular-weight copper complexes, are produced in the liver and secreted into plasma, increasing 2–3-fold as an acute phase response to infection [29]. This is consistent with the observation that copper complexes have antimicrobial activity in addition to hematopoietic activity [511]. Thus, major causes of death in the hematopoietic or bone marrow syndrome may be circumvented with the use of Cu(II)(3,5-DIPS)$_2$, as a radioprotectant. Since it has already been demonstrated that copper complexes increase the rate of re-epithelization in the denuded rat glandular stomach [178] and prevents erosions of the rat forestomach [22, 91, 267], in two different models of gastric ulcer, the use of Cu(II)(3,5-DIPS)$_2$ may aid in preventing radiation-induced epithelial mitotic arrest, denudation and ulceration leading to systemic infection, which may occur via the small intestine in association with any contribution of the gastrointestinal syndrome to death in irradiated mice. A positive antiulcer effect in the stomach or small intestine may impede fluid loss, haemorrhage, and electrolyte disturbances which are factors associated with lethality in this syndrome. The putative prodromal vascular and brain inflammatory changes associated with development of tremors, convulsions and ataxia prior to death in the cerebrovascular

or central nervous system syndrome may be another component of the radioprotectant activity of Cu(II)(3,5-DIPS)$_2$ when higher doses of radiation have been used, since this complex also has antiinflammatory and anticonvulsant activities [22, 324, 326, 515].

Copper complexes may also have the advantage of being relatively non-toxic. The LD_{50} values for Cu(II)(3,5-DIPS)$_2$ in female rats and mice were found to be 240 $\pm$ 33 mg/kg and 261 $\pm$ 89 mg/kg s.c., respectively, and its pharmacological effects have all been observed at much smaller doses ranging from1 to 30 μmol/kg [22, 91, 178, 267, 324, 326, 375–377, 511, 514, 515]. Additional evidence that copper complexes can be safely used in therapy comes from the work of German and French physicians who successfully treated patients with arthritis and other degenerative diseases with copper complexes without observing serious toxic effects [94] (see first section of this chapter also).

Radioprotectant studies of copper complexes with anticancer, anticarginogenic and antimutagenic activities as well as anti-inflammatory, antiulcer and anticonvulsant activities for use in protecting normal tissues of cancer patients undergoing radiotherapy appear to be fully justified.

INFECTIONS

It has been known for many years that the onset of inflammatory diseases may follow or occur along with a variety of infections. As a result, it is of interest that copper complexes have antiviral, antibacterial, antiprotozoal, anthelmintic, antifungal, antialgal and antimycoplasmal activities.

In the 1920s, it was recognized that the incidence of tuberculosis was much less in copper miners than in the general population. This led to the successful use of copper oxide (CuO) in the treatment of tuberculosis prior to 1940. In the 1940s, several additional copper complexes were reported to have antitubercular activity and it was suggested that one of them, the copper complex sodium 3-(allylcuprothiouredo)-1-benzoate (allocupreide sodium), was superior to gold for therapy of tuberculosis [523–525].

Subsequent research led to the discovery that *p*-aminosalicylic acid (PAS) was an active antitubercular agent, and it was suggested that the antitubercular activity of this drug was due to its Cu(II) complex [526], which was prepared and found to be 10-times as active as PAS itself [527]. Studies of Cu(II)(4-aminosalicylate)$_2$ also revealed that it was 30-times more lipid-soluble than PA [528, 529]. The enhanced activity of the copper complex was attributed to this increased lipid solubility, which facilitated penetration of the fatty outer membrane of *Mycobacterium tuberculosis*.

Similarly, additions of copper were found to enhance the *in vitro* activity of isonicotinylhydrazide (INH) [530]. This led to several demonstrations that the copper complex of INH was the active form of this antitubercular agent [531–533]. It was also found that the copper complex of a substituted hydrazide, 2-nitrobenzylidenoisonicotinoylhydrazide, was the most effective antitubercular complex in a study of 11 derivatives [534]. However, we appear to have taken a step backward. While copper complexes are known to have antitubercular activity in humans and the copper complex of INH is more effective than INH itself, the current drug of choice in the treatment of tuberculosis is INH and not its copper complex [535].

After considering the matter of copper complexation with INH, Krivis and Rabb [536] suggested that a 1:1 cuprous complex was more likely than a cupric complex. They pointed out that the copper complex formed with INH followed the reduction of Cu(II) to Cu(I), so that 1:1 cuprous complexes were formed upon addition of Cu(II) to solutions containing INH. Krivis and Rabb also suggested that the formation of Cu(I) complexes of thiosemicarbazones accounted for the synergistic effect of copper with the antitubercular agent 4-acetamidobenzaldehyde thiosemicarbazone (thiacetazone).

Copper complexes of thioureas and N-substituted thioureas have also been suggested to account for the effectiveness of the parent compounds as antitubercular agents [537]. On the other hand, Ueno [538] suggested that the parent thioureas acted by removing copper from some *M. tuberculosis* copper-dependent enzyme and that the antitubercular activity was dependent on the relative stabilities of the enzyme complex and thiourea complex. However, the activities of two series of N-substituted (2-pyridyl and 4-pyryl) thioureas [539] could not be correlated with copper complex stability [540].

In 1944, Albert, Gibson and Rubbo [541] found that Cu(II)(8-hydroxyquinoline)$_2$ was much more effective as a bactericidal and fungicidal agent than 8-hydroxyquinoline (8-HQ). Copper complexes of halo-substituted 8-HQ have been found by Gershon [542] to be even more effective than Cu(II)(8-HQ)$_2$. In addition, both 8-HQ [543] and its copper complex [544] have been found to have antiviral activity. It was suggested that the parent compound inhibited the RNA-dependent DNA polymerase of chick myeloblastosis virus and its copper complex produced the same effect with the RNA-dependent DNA polymerase of the tumourogenic Rous sarcoma virus.

Isatin-β-thiosemicarbazone was originally found to be a potent antivaccinia viral agent in mice and the activity of this compound was attributed to complex formation [545]. Subsequently, a derivative, *N*-methylisatin-3-thiosemicarbazone (M-IBT), proved to be a very effective antiviral agent in humans for the prevention of morbidity and mortality due to smallpox epide-

mics [546]. It was also found that addition of copper to M-IBT enhanced the *in vitro* inactivation of Rous sarcoma virus, as well as other RNA tumour viruses and herpesvirus, an effect that was later shown to be due to direct association of the copper complex with nucleic acids of Rous sarcoma virus rather than inhibition of the zinc-requiring RNA-dependent DNA polymerase [547, 548]. Later, it was suggested that this and other RNA tumour viruses were inactivated with the copper complex, but not M-IBT, as a result of bonding to single- and double-stranded DNA and RNA [544].

The enhancement of antibiotic activity has been extended to include many classes of complexing agents, including the naturally occurring antibiotics [541]. Doluizio and Martin [549] found that the more active tetracyclines formed 2:1 copper complexes of high stability, while the less active tetracyclines formed 1:1 complexes of lower stability. The antitubercular activity of streptomycin has been attributed to a 3:1 copper complex [528], since this complex, containing three copper atoms, provides higher blood levels of this drug for a longer time *in vivo*. It has also been pointed out that other well-known antibiotics such as penicillin and bacitracin are complexing agents [546].

The medical need for new wide-spectrum antibiotics led to the discovery of copper myxin, [Cu(II)(6-methoxy-1-phenazinol-5,10-dioxide)$_2$], which is very effective against Gram-positive and Gram-negative bacteria, fungi and yeasts [550]. Its activity *in vivo* and *in vitro* indicates that it is more effective than penicillin, streptomycin, nitrofurazone, oxytetracycline and sulfadimethoxine as an antibacterial agent and more effective than tolnaftate, nystatin and amphotericin B as an antifungal agent. Results obtained with this copper complex raise the question as to whether copper complexes of other synthetic or naturally occurring antibiotics might be more potent and effective as wide-spectrum antibiotics than the parent compounds.

Fluoropsin C [Cu(II)(*N*-methylthioformylhydroxamate)$_2$] has also been found to be very effective against Gram-positive and Gram-negative bacteria [551, 552]. Studies in mice indicated that the LD_{50} value:ED_{50} value ratio is large and, as a result, safe for drug use. It is also of interest that this complex was found to have potent *in vitro* antitumour activity.

Cuphen (Cu(II)(3,4,7,8-tetramethyl-1,10-phenanthroline)$_2$) has been suggested as being useful for the treatment of moniliasis (*Candida albicans*), pustular acne vulgaris, staphylococcal infections, and vaginitis due to either *C. albicans* or *Trichomonas vaginalis* [553].

Consistent with the above observations, many copper complexes reported to have anti-inflammatory and antiulcer activities [22] were also found to have antiviral activity against influenza A, strain 575 virus; antibacterial activity

against *Diplococcus pneumoniae, Escherichia coli*, and *Erwinia sp.*: antiprotozoal activity against *Tetrahymena gellii, T. vaginalis*, and *T. foetus;* anthelmintic activity against *Turbatrix aceti*; antifungal activity against *Trichophyton mentagrophytes* and *C. albicans;* and antialgal activity against *Chlorella vulgaris* in *in vitro* test systems [554].

Osterberg, Sjoberg and Branegard [555] have pointed out that liniments and salves prepared with dilute solutions of inorganic copper salts are also effective as fungicidal and bactericidal preparations. They are especially useful in treating scalp infections such as dandruff, seborrheic skin disease and warts, since they do not cause contact dermatitis or other allergic reactions. These authors also suggested wearing copper barrettes, chains or bracelets as an alternative to the use of liniments or salves to control fungal or bacterial skin afflictions. It was thought to be unlikely that absorption of copper from these devices, in the form of amino-acid complexes, would be harmful, since only small amounts of the element would be absorbed. Support for this suggestion has been abundantly provided [164, 165, 169–172].

Mycoplasmal infection may also play a role in the etiology of rheumatoid diseases [556, 557]. Since copper complexes of 1,10-phenanthroline, 2,9-dimethyl-1,10-phenanthroline, 1,3-disubstituted isoquinolines, and 2,2′-bipyridine have been shown to be effective antimycoplasmal agents [558–561], this approach to understanding the effectiveness of therapy and the physiological response associated with elevated blood copper levels in arthritic diseases also merits study.

Recent reports of antimicrobial effects for new copper complexes continues to be relevant to the treatment of rheumatoid and other infectious diseases with copper complexes. Halogen-substituted copper acetate complexes, $Cu(II)_2(XCH_2COO)_4$, and arylcarboxylates, $Cu(II)_2(2\text{-}XC_6H_4COO)_4$, where X = F, Cl, Br or I, were found to be effective against bacteria (*Staphylococcus aureus, E. coli,* and *Pseudomonas aeruginosa*) and fungi (*T. mentagrophytes* var. *granulosa* and *interdigitale, Microsporum gypseum* and *Epidermophyton floccosum*) [562].

Amoeba infections have also been pointed out as etiologic agents in rheumatoid and other connective tissue diseases and organic antiamoebic agents, including copper salts have been used to successfully treat these diseases [563].

All of these antibiotic activities may facilitate the PMN's ability to destroy micro-organisms and account for the decreased residence time of PMN-phagocytized bacteria following exposure to $Cu(II)_2(aspirinate)_4$ as compared with exposure to aspirin [564].

Copper(II)$(salicylate)_2$ was found to completely abolish an *E. coli*-lipopoly-

saccharide-induced fever in rabbits, while sodium salicylate produced only a 50% decrease in body temperature [192a, 565]. Cu(II)(salicylate)$_2$ also reduced normal body temperature 0.8 °C. The lipopolysaccharide-induced elevation in ear skin temperature fell 9.2 °C with no treatment, while Cu(II)(salicylate)$_2$ treatment decreased this fall in ear skin temperature to only 0.1 °C and sodium salicylate produced a decrease of 3.5 °C. These results suggest that Cu(II)(salicylate)$_2$ may have facilitated loss of body heat by maintaining ear skin vasodilatation more efficiently than sodium salicylate. Copper(II)(salicylate)$_2$ also prevented the pyrogen-associated reduction in respiratory frequency, another mechanism of facilitating body heat loss. It was suggested that copper modulates the thermoregulatory effects of salicylate; however, the effect of salicylate may be due to the formation of Cu(II)(salicylate)$_2$ *in vivo*. The more effective antipyretic activity of Cu(II)(salicylate)$_2$ was due to facilitated body heat loss by increasing skin temperature and respiration frequency.

The *E. coli* lipopolysaccharide-induced fever coincided with an increase in serum copper [566]. Treatment with Cu(II)(salicylate)$_2$ decreased serum copper and ceruloplasmin levels in febrile rabbits. The amount of copper in this complex was too small too cause any disturbances in metabolism of other essential metalloelements.

Schistosome egg granuloma development in the liver of infected mice was markedly inhibited by treatment of these mice with Cu(II)(3,5-DIPS)$_2$ [567]. Significant ($P < 0.001$) decreases in granuloma size and inflammatory halo were attributed to the potent SOD-mimetic activity of this complex. These results may also be due to facilitated formation of quality granuloma as a component of an enhanced immune response (p. 456).

BIOCHEMICAL AND PHYSIOLOGICAL ASPECTS OF COPPER COMPLEXES THAT SUPPORT POSSIBLE MECHANISMS OF ACTION

MODULATION OF LYSYL OXIDASES

It is well known that repair of tissues damaged as a result of inflammation, a consequence of many varied insults, requires cross-linking and extracellular repair and maturation of collagen and elastin. Since enzymes responsible for this, lysyl oxidases, are copper-dependent [568–572], this aspect of wound or tissue repair assumes particular significance. Lysyl oxidase activity is induced in copper-deficient chickens with copper(II) sulphate [571], and (+)-catechin

appears to function as a copper ionophore in this same model [573]. If lysyl oxidase is a soluble amine oxidase that requires pyridoxal, then the growing body of evidence that the cofactor for these enzymes is a pyridoxal-copper complex has added interest [574–584]. However, recent evidence suggests that the cofactor for some copper-dependent lysyl oxidases and for amine oxidases are not pyridoxal-dependent, but require some other carbonyl-containing cofactor, perhaps pyrroloquinoline quinone [585–587]. The copper dependency of both of these cofactors is of equal interest in accounting for the observed pharmacological effects of copper complexes.

This postulated mechanism for copper complex action is consistent with Townsend and Sorenson's [178] observations that the quantity and quality of replaced collagen in Cu(II)(tryptophanate)$_2$ and Cu(II)$_2$(aspirinate)$_4$-treated rats with surgically placed gastric lesions were superior and not readily distinguishable from normal in comparison with the 'scar-like' tissue replacement and adhesions found in non-treated controls. Recently reported stimulation of proteoglycan synthesis and maintenance of cartilage integrity by copper may also offer a partial accounting for these observations [588, 589].

MODULATION OF EICOSANOID SYNTHESES AND ARACHIDONATE METABOLISM

Modulation of prostaglandin syntheses [136] persists as an increasingly attractive mechanism of action for copper complexes. Inorganic copper salts as well as copper complexes have been shown to decrease the synthesis of the proinflammatory prostaglandin (PGE_2, vasodilator) and concomitantly increase the synthesis of the anti-inflammatory prostaglandin ($PGF_{2\alpha}$, vasoconstrictor) [271, 282–284, 590]. It is also of interest that rat hearts perfused with pg/ml to μg/ml concentrations of either PGE_2 or $PGF_{2\alpha}$ caused rhythm disturbances which were completely eliminated when a 2 μM solution of Cu(II)sulphate was added to the perfusate [591].

These results are consistent with the report that physiological concentrations (μM or less) of copper complexes rapidly inhibited prostaglandin-mediated mesenteric vascular responses to norepinephrine (noradrenaline) and angiotensin, which require intracellular calcium release, but did not inhibit potassium-mediated vascular responses, requiring extracellular calcium entry [286]. Antagonism of PGE_2, PGE_1 and thromboxane (TXA_2) vascular responses to norepinephrine and prostacyclin (PGI_2) agonistic activities of copper were interpreted as a role for copper as a 'second messenger' for PGI_2-mediated intracellular calcium release [286]. Modulation of syntheses of TXA_2 and PGI_2 as well as that of fatty acid syntheses [592] may also

bear on possible mechanisms of copper-complex-mediated decreases in vascular responses to norepinephrine. There would seem to be some necessary relationship between these observations and altered membrane and contractile properties of vascular tissues found in copper-deficient rats [572]. Studies of the influence of copper complexes on prostaglandin-mediated antisecretary, antiulcer, cytoprotective and diarrhoeogenesis [593] also shed some light on the mechanism of antiulcer and antisecretory actions of copper complexes. The significance of Cu(II)(cromoglycate)$_2$- and Cu(II)(3,5-DIPS)$_2$-mediated decrease in leukotriene syntheses [594] also awaits further study. All of these alterations of arachidonate metabolism may also have a bearing on the observation that Cu(II)(3,5-DIPS)$_2$ is more effective than Cu-Zn SOD in preventing an increase in vascular permeability as a result of reperfusion injury [595].

Active resorption of calcium from explanted neonatal mouse calvaria was inhibited by medium containing 1.0 μM Cu(II)sulphate [596]. This non-toxic concentration of copper(II)sulphate was more effective than Au(I)Cl, sodium aurothiomalate, (NaAu(I)thiomalate), and Zn(II)sulphate and NaAu(I) thiomalate and auranofin were toxic at effective concentrations [597]. These results indicate a possible mechanism for the prevention of bone resorption in relation to elevated serum copper associated with rheumatoid arthritis, a restoration of the normal rate of bone resorption [596]. This potent Cu(II)sulphate-induced inhibition of bone resorption was also related to enhancement of $PGF_{2\alpha}$, PGE_2, 6-keto-$PGF_{1\alpha}$ and TxB_2 contents of copper-treated calvaria [598]. The stimulation of bone resorption by exogenous prostaglandins was significantly ($P < 0.05$) inhibited in the presence of medium containing 5 μM Cu(II)sulphate. Copper also increased the inhibition of bone resorption observed with indomethacin ($P < 0.05$). This concentration of copper also decreased β-glucuronidase activity in the medium to 86% of the control value ($P < 0.001$). Incubation of calvaria with added arachidonic acid and copper resulted in a concomitant increase in PGF_2 and decrease in PGE_2 syntheses, along with a decrease in active resorption of calcium [598].

The observation that copper supplementation modulates the formation of arachidonic acid by regulating Δ^5-desaturase activity, which converts dihomo-γ-linolenic acid to arachidonic acid, has led to the suggestion that combinations of linoleic, γ-linolenic or dibromo-γ-linolenic acid with assimilable copper complexes might be useful in the treatment of inflammatory disorders, cardiovascular and thrombotic disorders, menstrual cycle disorders, diabetes, endometriosis, nutritional deficiencies and malignancies [255]. Modulation of stearoyl CoA Δ^9-desaturase activity by Cu(II)(tyrosinate)$_2$, Cu(II)(lysinate)$_2$, and Cu(II)(histidinate)$_2$ [599] may also have a bearing on these uses of copper complexes.

MODULATION OF SUPEROXIDE DISMUTASE AND SUPEROXIDE DISMUTASE MIMETIC REACTIVITY IN CHRONIC CONNECTIVE TISSUE DISEASES

The suggestion that superoxide produced by phagocytosing polymorphonuclear (PMN) leukocytes may account for the degradation of hyaluronic acid in synovial fluid of inflamed joints [106] stimulated research with regard to decreases in tissue Cu-Zn SOD and the role of superoxide in the etiology of rheumatoid and other connective tissue diseases. It was subsequently found that PMNs from juvenile rheumatoid arthritics produced more superoxide than PMNs from normal children [600, 601] and that Cu-Zn SOD activity was decreased in these cells when compared with that in normal PMNs. Stimulated PMNs produce oxygen radicals which disturb the immunocompetency of synovial fluid lymphocytes obtained from rheumatoid arthritic and systemic lupus erythematosis patients [602, 603]. However, PMNs from systemic lupus erythematosis patients produced less superoxide and released small amounts of lysosomal enzymes during immunosuppressive therapy while intracellular Cu-Zn SOD activity was unchanged [604]. Others found that superoxide production by PMNs obtained from rheumatoid arthritic and ankylosing spondylitis patients did not differ from normal control PMNs throughout a 30 minute incubation, and while Cu-Zn SOD activities were not different from normal controls, the Mn SOD was markedly decreased ($P \leq 0.025$) for PMNs from both patient populations [605, 606]. There was no significant correlation between Cu-Zn SOD, Mn SOD or superoxide production for normal PMNs, while superoxide production by PMNs from rheumatoid arthritics significantly correlated with Cu-Zn SOD ($r = 0.5$, $P = 0.025$) and superoxide production by ankylosing spondylitis patients correlated with Mn SOD ($r = 0.88$, $P < 0.001$) [605, 606]. Superoxide formation by resting PMNs from patients with juvenile rheumatoid arthritis was normal, while stimulated (phagocytosing) PMNs produced much greater amounts of superoxide. PMNs from a child with chronic granulomatous disease produced less superoxide while at rest than during phagocytosis, when production was also stimulated [607]. PMNs from control (normal individuals) and from systemic lupus erythematosis patients produced similar amounts of superoxide whether at rest or during active phagocytosis [604]. Immunosuppressive therapy produced a marked decrease in superoxide production at rest and during phagocytosis [604], and a marked decrease in lysosomal enzyme release by stimulated PMNs.

It is also of interest to note that red blood cells from rheumatoid arthritis patients were found to have less than normal Cu-Zn SOD [74] and the decrease in Cu-Zn SOD significantly correlated with increased serum thiol,

increased erythrocyte sedimentation rate, decreased haemoglobin, decrease in red blood cells, and an increase in arthritic disease activity [74]. If red blood cell number is not taken into account, then the RBC Cu-Zn SOD activity may appear to be similar for rheumatoid arthritis patients and normal individuals [608]. This is particularly noteworthy since Cu-Zn SOD activity has been shown to depend on dietary copper intake [608–612] and copper deficiency causes a decrease in number of red blood cells [611].

A remarkable increase in SOD-mimetic activity was found in a comparison of synovial fluid from rheumatoid arthritis and osteoarthritic patients with normal control values [613]. The increase in SOD-mimetic activity correlated with increased rheumatoid disease activity and increasing progression of disease severity. There was also a good correlation between SOD-mimetic activity and C-reactive protein in synovial fluid from patients with rheumatoid arthritis. This SOD-mimetic activity may be attributed to either an elaboration of ceruloplasmin by synovial cells [614] or the liver along with copper albumin and amino-acid copper complexes which, in part, accounts for the established increase in synovial fluid copper and ceruloplasmin in rheumatoid arthritis [30] and it is well known that ceruloplasmin [102, 489, 615] as well as amino-acid and other small-molecular-weight copper complexes have SOD-mimetic activity [287–295, 327].

The *in vitro* anti-inflammatory effects of Cu-Zn SOD are now attributed to the prevention of hyaluronic acid depolymerization [106] and the prevention of chemotactic factor elaboration as a result of PMN produced superoxide [616–619]. However, the modest *in vivo* anti-inflammatory activity of Cu-Zn SOD in a variety of inflammatory diseases of man [620–628] has not been encouraging. Its lack of effectiveness is most likely due to its very short half-life (about 6 min), which may arise from the antigenicity of this intracellular enzyme in extracellular spaces, and its inability to cross cell membranes and protect intracellular targets of oxygen radicals due to its large mass and polar character.

Copper complexes of all anti-inflammatory drugs studied to date have been found to have SOD-mimetic activity. Many copper complexes including those antiarthritic drugs, disproportionate superoxide at essentially the same rate as Cu-Zn SOD [194, 197, 287–295, 591, 629–643]. It has been suggested that superoxide-derived oxygen radicals such as singlet oxygen, hydroperoxyl, and hydroxyl radicals may account for inflammogenic tissue damage [444, 602, 603, 607, 613, 644]. Since small-molecular-weight copper complexes capable of crossing cellular and other lipid barriers also disproportionate superoxide and hydroperoxyl radical, reduce hydroxyl radical, and react with aquated electrons at diffusion controlled rates [518–520], they offer many advantages

over Cu-Zn SOD in therapy. The SOD-mimetic activity of $Cu(II)_2$(aspirinate)$_4$, Cu(II)(3,5-DIPS)$_2$ and $Cu(II)_2$ (indomethacin)$_4$ has also been used to account for the greater effectiveness of these complexes in modulating chemotaxis than their parent ligands [164a]. Finally, the peroxidase-mimetic and catalase-mimetic activities of copper complexes [645] as well as their myeloperoxidase-mimetic activity [645a], in particular Cu(II)(3,5-DIPS)$_2$ [645, 645a], offer still other mechanistic advantages over Cu-Zn SOD therapy of inflammatory diseases including: arthritides epilepsies, ulcers, cancers, carcinogeneses, mutageneses, diabetes, radiation injuries and reperfusion injuries.

STABILIZATION OF SYNOVIAL AND POLYMORPHONUCLEAR LEUKOCYTE LYSOSOMAL MEMBRANES

Another possible biochemical mechanism of action for copper complex action is based upon the report that copper decreased the permeability of human synovial lysosomes obtained from arthritic patients by oxidizing membrane thiols to disulphides and, as a result, decreased the release of free lysosomal enzymes [80]. Membrane stabilization as opposed to lysosomal enzyme inhibition is also consistent with the observation that many copper complexes, with the exception of Cu(II)(niflumate)$_2$, failed to inhibit cathepsin-D, a lysosomal proteinase [647].

Stabilization of lysosomes in PMNs with copper was suggested to account for the reversal of diethyldithiocarbamate-induced PMN toxicity [648]. In the observed biphasic toxic response, copper protected against the second phase, which was suggested to be due to a re-establishment of the demonstrated loss of Cu-Zn SOD activity. Zinc was more effective in protecting against the first phase of toxicity, which was suggested to be due to the loss of enzymatic activity associated with some zinc-dependent enzyme. Since Cu-Zn SOD contains both zinc and copper, the first phase of toxicity may be associated with the removal of zinc from Cu-Zn SOD, while the second phase may be associated with the removal of copper from this enzyme.

MODULATION OF HISTAMINIC ACTIVIY

Evidence supporting the suggestion that a copper complex of histamine might be the active form of histamine has been published [20, 649, 650]. Agonist anaphylactic activity of Cu(II)(histamine)$_2$(OH)$_2$ was reported to be greater than that found for the parent ligand alone. The activity of the complex could be decreased by administering zinc salts or a chelating ligand such as sodium salicylate. These results seem to be consistent with the demonstration that

Zn-(lidocaine)$(Cl)_2$ and Zn-(H-lidocaine)$_2(Cl)_2$ complexes inhibited compound 48/80-induced histamine 'release' from rat mast cells [651]. These complexes also inhibited ionophore A23187- and X537A-induced 'releases' of histamine from mast cells. Effects of the corresponding copper complexes were reported to be 'weaker' than the zinc complexes and the Cu-(H-lidocaine)$_2(Cl)_2$ complex 'increased' histamine release [652]. It was subsequently reported that the Cu(II)(histamine)(Cl)(HCl) complex and histamine were equiactive in *in vitro* tests, but the copper complex was always, but not significantly, more active than histamine [653]. Vascular permeability and oedema responses were also similar. In addition, coadministration of ascorbic acid markedly potentiated the effect of the copper complex, while the action of histamine was unchanged. This suggested a role for cuprous copper in modulating histaminic effects, since ascorbic acid reduces cupric copper to cuprous.

However, copper(II)(histamine)$(Cl)_2(HCl)_2$ has subsequently been found to be a potent antihistaminic compound having antiulcer and antisecretory activity [22, 267]. These and other data (p. 491) suggest that physiological levels of copper complexes have antihistaminic activity.

MODULATION OF LYMPHOCYTE MITOGENIC AND CHEMOTACTIC RESPONSES

Preincubation of human T-lymphocytes with D-penicillamine (333 μM) and inorganic copper salts (13 μM Cu(II)sulphate) decreased their responsiveness (DNA synthesis) to mitogens [654]. While mixtures of penicillamine and $CuCl_2$, $CuSO_4$, Cu(II)$_2$(acetate)$_4$ [654], or ceruloplasmin [655] were effective in decreasing mitogen responsiveness, mixtures of penicillamine and Ca(II)(chloride)$_2$, Fe(II)sulfate, Au(III)(chloride)$_3$ or Mn(II)(chloride)$_2$ had no inhibitory capacity. Other mercaptans, such as *N*-acetyl-L-cysteine, cysteamine, D-cysteine, L-cysteine, 2-mercaptoethanol or DL-thiomalic acid were as effective as penicillamine when mixed with inorganic copper salts. As a result, it was suggested that the mechanism of action of penicillamine in the treatment of rheumatoid arthritis involves *in vivo* formation of penicillamine copper complexes with the capacity to decrease T-lymphocyte function [654]. Combinations of penicillamine and copper did not alter the capacity of monocytes to act as accessory cells in response to mitogens [654]. Coculturing penicillamine and Cu(II)sulphate-treated T-cells with monocytes reversed this inhibition of T-cell activity, indicating protection by monocytes.

Other thiols used to treat rheumatoid arthritis, 5-thiopyridoxine and 2-mercaptopropionylglycine, in combination with Cu(II)sulphate were also found to inhibit helper T-cell activity [655, 656] by inhibiting the generation of immuno-

globulin-producing cells rather than β-cell differentiation to these cells [657–659]; this was suggested to provide further support for the immunosuppressive efficacy of penicillamine in the treatment of arthritis.

It was also found that inhibition of both T-lymphocytes and T-helper cells by penicillamine and Cu(II)sulphate or ceruloplasmin could not be blocked by Cu-Zn SOD but was completely blocked by catalase, suggesting mediation of inhibition by hydrogen peroxide [655]. Mononuclear phagocytes, which were not inhibited by the combination of penicillamine and Cu(II)sulphate, were found to protect T-lymphocytes from this mixture as well as hydrogen peroxide, and the protective factor was found to be a soluble fraction of human monocyte or erythrocyte lysates.

These observations have been confirmed [660–662] and appear to be related to the addition of non-physiological forms of copper which catalyze the oxidation of penicillamine to its disulphide wherein oxygen is consumed in the production of superoxide [660] and hydrogen peroxide or singlet oxygen [444]. These results await further clarification, as it was found that penicillamine or cysteine reversed hydrogen peroxide-mediated inhibition of mitogen-stimulated mononuclear cell proliferation [661] and visible light may cause a homolytic cleavage of thiols to yield hydrogen atoms capable of generating superoxide [663] and singlet oxygen [444]. The above observations are also consistent with the possibility that T-lymphocytes and T-helper cells utilize glutathione peroxidase to metabolize hydrogen peroxide and the high concentrations of Cu(II)sulphate (13 μM) used in these experiments exceed the concentration (1 μM) known to inhibit this enzyme system [664].

While it seemed reasonable to suggest that copper penicillamine complexes might mediate these events, Cu(I)(penicillamine), $Na_5[Cu(II)_6Cu(I)_8(penicillamine)_{12}Cl]$, and $Cu(II)_2$(penicillamine disulphide)$_2$ as well as $Cu(II)_2$(cystine)$_2$ did not inhibit T-lymphocyte function [665]. Addition of Cu(II)sulphate to medium containing these complexes also did not produce inhibition, while addition of penicillamine (50 μg/ml) did inhibit PHA-induced [^{3}H]thymidine incorporation. These complexes appeared to fulfil requirements for copper, but additional penicillamine was required, as had been found for the inhibiting mixture of penicillamine (333 μM) and Cu(II)sulphate (13 μM). This excess penicillamine could interfere with glutathione peroxidase by decreasing the oxidation of reduced glutathione, causing the formation of mixed disulphides, or inhibiting glutathione disulphide reductase in preventing glutathione-dependent reduction of peroxide.

The copper chelate of albumin, which has copper bonded to a specific bonding site (histidine at position 3), has been shown to be a potent non-cytolytic antiproliferative agent [666]. This complex decreased phytohaemag-

glutinin-M (PHA), lipopolysaccharide and allogenic cell-induced proliferation of mouse splenocytes and PHA proliferation of human leukocytes. Suppression of lymphocytes occurred in both G_1 and S phases of the cell cycle. Albumin without copper had no effect in a competitive inhibition study and copper-albumin added during the S phase of the cell cycle rapidly stopped DNA synthesis without suppressing protein synthesis ([^{3}H]leucine incorporation) or causing cell death (trypan blue exclusion). A high concentration of Cu(II)sulphate (1×10^{-4} M) was effective in suppressing the PHA-colchicine response, but copper-albumin was 200-times more effective in causing suppression, achieving 50% suppression at 2 μM, with an excellent correlation ($r = 0.94$) between increasing suppression and increasing copper saturation of albumin, without interfering with [^{3}H]thymidine transport across cell membranes. Other albumins with histidine at the 3rd position were also potent suppressors when combined with copper. No immunosuppressive activity was observed with ceruloplasmin. It was suggested that the well known immunosuppressive activity of amniotic fluid may be due to a copper complex of albumin or a copper complex of α-fetoprotein, which also contains a 3-histidine.

Effects of penicillamine and Cu(II)sulphate or the mixed valence copper complex of penicillamine, $Na_5Cu(I)_8Cu(II)_6$(D-penicillamine)(Cl), on migration of rat leukocytes have also been studied [667]. Penicillamine or Cu(II)sulphate caused only a slight suppression of migration of this mixed population of leukocytes, while the addition of Cu(II)sulphate to penicillamine appeared to decrease the slight inhibition of mononuclear cell migration found with penicillamine alone and the greater inhibition of PMN migration found with Cu(II)sulphate alone decreased with the addition of penicillamine. However, the mixed-valence copper complex alone produced a marked inhibition (90%) of both mononuclear and PMN chemotaxis [667]. This investigation also supported the observation [656] that the combination of penicillamine and Cu(II)sulphate did not inhibit phagocytic activity and the residence time of PMN-phagocytized bacteria was shortened in $Cu(II)_2$(aspirinate)$_4$-treated cultures in comparison with that of aspirin-treated cultures [564]. Results of this study were confounded by the observation that the concentration of 10 μg/ml of the mixed valence complex decreased viability to 13% and 75% for mononuclear and polynuclear cells, respectively, in parallel cultures when it had been earlier observed that 30 μg/ml did not affect viability.

THERMAL STABILIZATION OF HUMAN GAMMA-GLOBULIN

Heat-denatured IgG is inflammatory when fixed with complement. Gerber was the first to report that a mixture of penicillamine disulphide and Cu(II)sul-

phate prevented heat-induced denaturation of human gamma-globulin [617]. Inhibition of synovial fluid gamma-globulin denaturation by a copper penicillamine complex was suggested as a plausible mechanism for the suppression of rheumatoid arthritis. It was subsequently found that penicillamine, cysteine, glutathione, histidine and their Cu(II)sulphate mixtures inhibited heat denaturation of gamma-globulin [669]. The effective concentration (EC) for Cu(I)/(II)-penicillamine (9.1 μM) was seven times lower than the value obtained for penicillamine (EC_{50} = 64.5) alone. Histidine was ineffective at 200 μM while the EC_{50} value for Cu(II)(histidine)$_2$ was 1.14 μM. The EC_{50} values for cysteine and glutathione were 131.4 and 17.45 μM, respectively, while Cu(I)/(II)-cysteine and Cu(I)/(II)-glutathione had EC_{50} values of 11.8 and 0.07 μM respectively. Zn(II)-penicillamine had no activity. Denatured IgG-complement complex may initiate PMN chemotaxis to the site of inflammation and cause superoxide mediation of tissue damage in chronic inflammatory disease. These results were interpreted as indicating that copper complexes might be effective in removing superoxide in successfully treating inflammatory diseases.

Studies of the reversal of copper-mediated aggregation of human IgG by nonsteroidal anti-inflammatory agents (NSAIA) were performed to determine inhibition of aggregation by nonsteroidal anti-inflammatory drugs. This effect was related to the supposed role of NSAIA removal of copper from synovial fluid of rheumatoid arthritis patients by forming a copper-NSAIA complex [670]. Steroids were not only ineffective in inhibiting aggregation, but promoted aggregation. Penicillamine and NaAu(I)(thiomalate) reversed aggregation and produced 50% inhibition at concentrations of about 50 and 150 μM, respectively. Indomethacin, phenylbutazone, aspirin, flufenamic acid, ibuprofen and sodium diclofenac appeared to be ineffective in reversing aggregation of copper aggregated human IgG under the same conditions of pH and solubility used for penicillamine and NaAu(I)(thiomalate). These results are consistent with the notion that the reversal of copper aggregation of IgG requires the drug to compete for the copper, which may be Cu(I), in the IgG copper complex in reversing aggregation and may only be possible when the competing ligand can form a stable Cu(I) complex, as in the case of penicillamine and thiomalate (*vide infra*).

PHARMACOKINETICS OF D-PENICILLAMINE AND COPPER IN RHEUMATOID ARTHRITIS

Studies of the interaction of administered copper (5 mg/day) and penicillamine (500 mg/day) have provided evidence that the penicillamine complex

formed *in vivo* may be a Cu(I)-(penicillamine) or Cu(II)-(penicillamine)(cysteine) complex rather than the mixed-valence complex, as a result of removal of copper from ceruloplasmin leaving apoceruloplasmin and causing an interference with its role in the physiological defence or response to inflammation [671]. With this evidence it can now be reasonably suggested that penicillamine's disease-modifying character may be devastating to the patient [672–674] and it is time to reconsider the use of penicillamine to treat connective tissue diseases.

Attempts to produce a SOD-mimetic copper complex of penicillamine *in vivo* in an effort to overcome rheumatoid arthritis [671], while meritorious in concept, is discounted in the light of the observation that penicillamine therapy decreases Cu-Zn SOD activity [672]. While this has been demonstrated using erythrocytes from patients with amyloidosis, it is likely that lowering of Cu-Zn SOD activity in still other cells of treated patients would further compromise the physiological response to these diseases and may account for the toxicity of this drug. Most of the side-effects of penicillamine therapy are rash and gastrointestinal upset [675]. It is noteworthy that this mixed valence complex of penicillamine has been shown to be the most effective of the penicillamine complexes as an antiulcer agent [91] and Scheinberg (Albert Einstein College of Medicine, Yeshiva University) has obtained Food and Drug Administration approval for oral use of the mixed valence copper complex of penicillamine to treat early rheumatoid disease. This complex would also be of value in treating juvenile rheumatoid arthritis and other connective tissue diseases.

PENICILLAMINE, THIOMALATE AND SODIUM GOLD THIOMALATE MODULATION OF COPPER METABOLISM

Munthe and Jellum [676] recognized that clinical responses and some side-effects are strikingly similar when penicillamine and NaAu(I)thiomalate are used in therapy of rheumatoid arthritis. They suggested that the gold of the thiomalate complex could be transferred to another thiol group *in vivo*, and demonstrated that serum albumin has a very high affinity for gold, as did other serum proteins, and that exchange occurs with the liberation of thiomalate. Studies of thiomalate and penicillamine revealed that they had similar effects in animal studies.

Intramuscular [676] or intraperitoneal [677] injection of rats with $Na^{195}Au(I)[^{14}C]$thiomalate demonstrated that these labels were quickly separated and excreted at different rates. Kidney and liver metallothioneine contained a large portion of ^{195}Au as did blood. It was remarkable that only

0.1% of the thiomalate was found in blood 6 h after injection, indicating very rapid tissue uptake. Brain, muscle and skin also had larger amounts of thiomalate than gold 6 h after injection. Labelled [^{14}C]penicillamine was similarly distributed to tissues and urinary excretion was similar, but thiomalate disappeared from blood and was taken up by tissues more rapidly than penicillamine. In *in vitro* studies, it was found that erythrocytes and liver cells were poorly penetrated by thiomalate as compared with penicillamine and there was no penetration of these cells by gold or copper complexes of these thiols.

Sharma and McQueen [678] studied liver and kidney cytosolic changes in copper following treatment of normal rats with a single subcutaneous injection of NaAu(I)thiomalate. Both high- and low-molecular-weight protein bonded copper increased in the liver cytosol. Copper increased in the low-molecular-weight protein and decreased in the high-molecular-weight kidney cytosolic proteins. There was no significant change in total kidney content of copper, suggesting that gold displaced copper from high-molecular-weight proteins, resulting in an intracellular redistribution to the low-molecular-weight copper bonding protein. Since gold and copper both induce biosynthesis of metallothioneine in rat kidney, it is likely that this induction was caused by the injected gold which also displaced copper from high-molecular-weight proteins. These results are also consistent with a copper-conservation role for the kidney and support the hypothesis that beneficial effects of gold complexes in the treatment of rheumatoid arthritis are due to their mobilization of stored copper.

Repeated subcutaneous injections of NaAu(I)thiomalate produced similar effects with regard to bonding of gold and copper to cytosolic proteins in liver and kidneys [679]. Liver gold increased with the first six injections and stabilized at about 1 μg/g of tissue as a steady state content. Kidney gold continued to increase with each injection and reached 24.8 μg/g after the 11th and final injection. Cu-Zn SOD fractions increased significantly, while the uptake of copper into high-molecular-weight ($> 60{,}000$) proteins decreased in liver and kidney cytosols. Copper levels in Cu-Zn SOD and the low-molecular-weight (< 4000) protein fractions initially increased and then decreased from the sixth injection onward, possibly due to a redistribution of liver copper. Incorporation of copper into hepatic metallothioneine was unchanged. Uptake of copper was significantly increased in the kidney metallothioneine fraction, but decreased in other fractions throughout the multiple dose period. These injections of NaAu(I)thiomalate caused an increase in kidney and a decrease in liver copper. Practically all additional copper in kidneys was incorporated into the metallothioneine fraction [679].

It was suggested that these observations indicated that NaAu(I)thiomalate has a major role in providing a stimulus for the liver, kidney and other cells to bring about a redistribution of body essential metalloelements, including copper [679]. The various cytosolic proteins, including the inducible metalloproteins, Cu-Zn SOD, and copper-thioneine, seem to help the cell carry out this task. With regard to the importance of copper in the physiological response to rheumatoid arthritis, it was suggested that gold complexes mediate their antiarthritic activity through effects on essential metalloelement metabolism and in particular copper metabolism in stimulating *de novo* synthesis of Cu-Zn SOD.

MODULATION OF GLUTATHIONE STATUS

Copper-induced decreases in yeast glutathione reductase [664] were used to suggest that the elevation in plasma copper is etiological for rheumatoid arthritis [680] in perturbing synovial cell metabolism and triggering this disease [681]. While addition of a non-physiological form of copper, Cu(II)sulfate (0.16 μg/ml of medium) did decrease the yeast reductase *in vitro*, doses of Cu(II)sulphate ranging from 0.8 to 6.4 μg/kg of body weight given by intraportal injection did not inhibit the rat liver glutathione reductase system [682]. Treatment with $Cu(II)_2(succinate)_2$, $Cu(II)_2(indomethacin)_4$, $Cu(II)(salicylate)_2$, $Cu(II)_2(aspirinate)_4$, or $Cu(II)(tyrosinate)_2$ generally increased glutathione levels, which was suggested to be due to superoxide disproportionation by these copper complexes and, as a result, the prevention of superoxide-mediated shift to glutathione disulphide [682]. Any conclusion based solely upon an effect of inorganic compounds of copper in a biological system can only be tenuous at best, since the normal concentration of ionic copper in biological systems is of the order of 10^{-18} M [5].

MODULATION OF GLUTATHIONE *S*-TRANSFERASE

The effect of copper complexes on changes in liver and intestinal glutathione *S*-transferase activities in oleyl alcohol paw oedema and adjuvant arthritis inflammations have also been studied [683]. Enzymatic activity was found to be unchanged in acute inflammation but decreased in chronic adjuvant arthritis. Because serum copper concentration is elevated in polyarthritis, $Cu(II)(glycylglycinate)_2$ was added to tissue homogenates to study the influence of copper on the amount of transferase activity. This complex further decreased the level of transferase activity, which was suggested to be the result

of an equilibrium shift from glutathione to disulphide. The formation of a Cu(I)-glutathione complex could also explain the decrease in transferase activity.

MODULATION OF PREPROTHROMBIN CARBOXYLASE

The nicotinamide adenine dinucleotide-dependent (NADH-dependent) generation of superoxide by normal rat liver microsomes is stimulated by the addition of vitamin K and decreased by cytosolic Cu-Zn SOD, which scavenges superoxide [684]. The vitamin-K-dependent carboxylation of preprothrombin, the precursor of prothrombin, or a pentapeptide composed of amino acids 5–9 of prothrombin is decreased by Cu-Zn SOD, but only at high concentrations. This inefficiency of Cu-Zn SOD in preventing carboxylation was explained as an inability of Cu-Zn SOD to reach the superoxide-generating site [685]. In an attempt to penetrate barriers to this site, low-molecular-weight SOD-mimetic copper complexes were tested. Copper(I)/(II)-penicillamine, Cu(II)(L-tyrosinate)$_2$, and Cu(II)$_2$(aspirinate)$_4$ [685, 686] as well as Cu(II)(chloride)$_2$ [686] were found to be more effective than Cu-Zn SOD in inhibiting this carboxylation. Alternatively, it was suggested that these low-molecular-weight copper complexes inhibited the carboxylating system by oxidizing vitamin K hydroquinone to the inactive quinone [686] and thus prevent the conversion of preprothrombin to prothrombin. These results point out a possible antithrombotic effect for copper complexes with SOD-mimetic activity.

MODULATION OF CYTOCHROME *P*-450 MONO-OXYGENASES AND/OR NADPH-DEPENDENT CYTOCHROME *P*-450 REDUCTASES

Copper-Zn SOD and low-molecular-weight copper complexes with SOD-mimetic activity have been shown to modulate superoxide-dependent biochemical processes. These biochemical processes appear to involve either copper-dependent or iron-dependent activation of oxygen wherein the oxygen-copper or oxygen-iron enzyme complex dissociate to yield superoxide or competition for this partially activated oxygen yields superoxide which undergoes disproportionation catalyzed by Cu-Zn SOD or copper complexes. On the other hand, small-molecular-weight copper complexes can act as electron acceptors and take up the reducing equivalents provided by vitamin C or a companion reductase, required for further activation of oxygen in the oxygen-copper or oxygen-iron complexes of these mono-oxygenases. In this regard, copper complexes are being used as probes to study the mechanism of various copper or iron dependent mono-oxygenases.

Modulation of drug metabolizing *P*-450 mono-oxygenases by copper complexes has been firmly established by Weser, Richter, Azzi, Wendel and Younes [291]. They were the first to demonstrate that liver microsomes from phenobarbital-induced rats did not dealkylate aminopyrine, *p*-nitroanisole, or 7-ethoxycoumarin when they were exposed to Cu(II)(tyrosinate)$_2$, Cu(II)(salicylate)$_2$, Cu(II)$_2$(aspirinate)$_4$, or Cu(II)(3,5-DIPS)$_2$ [291], established SOD-mimetic compounds [291, 687]. It was suggested that this decrease in activity was due to disproportionation of superoxide from oxycytochrome *P*-450. However, Werringloer, Kawano, Chacos and Estabrook [392] found that hydrogen peroxide was not produced in microsomal preparations containing benzphetamine or ethylmorphine and treated with Cu(II)(tyrosinate)$_2$. Similarly, Cu(II)(salicylate)$_2$, Cu(II)$_2$(aspirinate)$_4$, Cu(II)(3,5-DIPS)$_2$ or Cu(II)sulphate decreased the dealkylation of benzphetamine without producing hydrogen peroxide, a product of superoxide disproportionation. Subsequent studies revealed that these copper complexes impaired the NADPH-dependent cytochrome *P*-450 reductase by competing with the oxycytochrome *P*-450 intermediate as reducible substrates for the reductase [392]. The question still remains as to whether or not some complexes reach the juxtapositioned reductase and oxycytochrome complex site and either remove superoxide by disproportionation or remove reducing equivalents by oxidation of NADPH. Careful experimentation is required to answer this question, since spin-trapping experiments have shown that superoxide is the product of NADPH–cytochrome *P*-450 reductase reduction of oxygen [688].

Subsequent studies in rabbit microsomal preparations also demonstrated that Cu(II)(tyrosinate)$_2$, Cu(II)(lysinate)$_2$, and $CuCl_2$ decreased dealkylation of dimethylanaline [689].

Copper complex modulation of steroid hydroxylations have also been reported [690]. A single dose of Cu(II)sulphate injected into rats caused a 2- to 3-fold increase in adrenal copper, a significant ($P < 0.05$) increase in mitochondrial *P*-450-dependent steroid 11-β-hydroxylase activity, and an 85% increase in concentration of *P*-450 as well as a 3-fold increase in δ-aminolevulinate synthetase, which suggests a positive feedback stimulation of *P*-450 synthesis. Chronic treatment with Cu(II)sulphate for 7 days caused a decrease in 11-β-hydroxylase activity ($P < 0.05$) and a decrease in plasma corticosterone concentration ($P < 0.05$) while there was a concomitant increase in cholesterol side-chain cleavage, suggesting the metabolism of cholesterol by some other *P*-450 following maximal corticosterone synthesis. A positive feedback for δ-aminolevulinate synthesis was also evidenced by an increase in synthetase activity.

Copper(II)(salicylate)$_2$ was also reported to decrease the activity of

dopamine-β-hydroxylase, a copper-dependent enzyme [691]. It was also suggested that this reduction in activity was the result of superoxide disproportionation. The fact that Cu-Zn SOD did not inhibit this mono-oxygenase does not eliminate superoxide disproportionation as the mechanism of inhibition, since only lipid-soluble small-molecular-weight copper complexes may fulfill requirements for fit into the site of oxygen activation.

MODULATION OF 2-OXOGLUTARATE-DEPENDENT HYDROXYLASES

Conversions of peptide-prolyl and peptide-lysyl residues to hydroxyprolyl and hydroxylsyl residues are accomplished by iron-dependent enzymes utilizing 2-oxoglutarate, oxygen and ascorbate. Evidence that a superoxide intermediate is involved in this catalysis was provided when it was shown that SOD-mimetic complexes including $Cu(II)_2(aspirinate)_4$, $Cu(II)(salicylate)_2$, $Cu(II)(lysinate)_2$, and $Cu(II)(tyrosinate)_2$ decreased these conversions [692]. Similarly, $Cu(II)(salicylate)_2$ and Cu(II)sulphate were shown to be as effective as Cu-Zn SOD in decreasing the ascorbate- and 2-oxoglutarate-dependent γ-butyrobetaine hydroxylase [693]. Whether or not these inhibitions were due, in part, to the oxidation of ascorbate by these complexes is still unclear.

MODULATION OF DIOXYGENASES

Both protocatechuate 3,4-dioxygenase and dihydroxyphenylacetate 2,3-dioxygenase activities were decreased by $Cu(II)(salicylate)_2$ but not Cu-Zn SOD, supporting the notion that small-molecular-weight and lipophilic SOD-mimetics were able to reach the site of superoxide generation [694]. Similarly, a Cu(I)(chloride) pyridine solvate was found to decrease the tryptophan 2,3-dioxygenase activity [695].

MODULATION OF LIPID PEROXIDATION

Copper deficiency is reported to cause a 2-fold increase in the level of lipid hydroperoxides in rat liver mitochondria and microsomes along with decreases in Cu-Zn SOD [696]. Cytosolic and mitochondrial glutathione peroxidase and catalase activities were also decreased [696]. Increased lipid peroxidation was suggested to be due to the accumulation of hydrogen peroxide rather than superoxide. Alternatively, some lipid peroxidation may be due to the formation of singlet oxygen [444] and its oxidation of glutathione to its disulphide and/or oxidation of Fe(II)catalase to the inactive Fe(III) form.

It is known that liver microsomal lipid peroxidation (malondialdehyde formation) as well as drug metabolism are altered in relation to cytoplasmic copper content [697]. NADPH-dependent microsomal lipid peroxidation was decreased by appropriate addition of supernatant. Copper-Zn SOD had no effect on lipid peroxidation, whereas ceruloplasmin was inhibitory. In addition to inhibiting lipid peroxidation, copper in the supernatant also facilitated microsomal ethylmorphine demethylase activity by preventing peroxidative microsomal membrane damage due to the addition of NADPH-regeneration system [697]. This lipid peroxidation was suggested to be associated with NADPH reduction of oxygen to superoxide and self-disproportionation to singlet oxygen, with destruction of polyunsaturated fatty acids such as arachidonic acid and destruction of cytochrome *P*-450.

Small-molecular-weight copper complexes also inhibit lipid peroxidation. The oxidation of linolenic acid by the xanthine oxidase-acetaldehyde superoxide generating system was prevented by $Cu(II)_2(lonazolac)_4$ [194]. This complex was also effective in inhibiting linolenic acid oxidation in the presence of 0.9 mg/ml albumin or whole serum protein. At a concentration of 0.7 mM albumin, a ternary complex was formed with $Cu(II)_2(lonazolac)_4$ which also inhibited lipid peroxidation, as did $Cu(II)(salicylate)_2$ and $Cu(II)_2(indomethacin)_4$ [194].

Copper chelates have also been found to inhibit superoxide-induced lipid peroxidation in other *in vitro* systems. $Copper(II)_2(aspirinate)_4$, Cu(I)/(II)-penicillamine, and $Cu(II)_2(indomethacin)_4$ were as effective as or more effective than Cu-Zn SOD in inhibiting erythrocyte membrane and liver microsomal membrane peroxidation [636]. $Copper(II)(tyrosinate)_2$ was also found to be a very efficient inhibitor of NADPH-dependent lipid peroxidation in phenobarbital-induced rat liver microsomes [698]. Even though Cu-Zn SOD was ineffective in inhibiting this peroxidation, it was suggested that superoxide and other radicals participated in this NADPH-dependent peroxidation of microsomal unsaturated fatty acids and that these radicals react in domains of the microsomal membrane that are inaccessible to Cu-Zn SOD. The small size and lipophilic character of $Cu(II)(tyrosinate)_2$ allow penetration into these domains and the prevention of peroxidation.

Copper complexes also prevent lipid peroxidation *in vivo*. Treatment of benzo[*a*]pyrene-induced and paracetamol-dosed mice with $Cu(II)(tyrosinate)_2$, $Cu(II)_2(aspirinate)_4$, or Cu(II)EDTA, which also has SOD-mimetic activity [700], markedly decreased paracetamol (acetaminophen)-induced lipid peroxidation, gave a 97% decrease in ethane exhalation, and produced a glutathione-sparing effect, whereas Cu-Zn SOD had no inhibitory effect [699].

A single dose of Cu(II)sulphate injected into rats, sufficient to cause a 2- to 3-fold increase in adrenal copper content, produced a 50% reduction in mitochondrial lipid peroxidation as well as a statistically significant ($P < 0.05$) increase in cytochrome *P*-450, providing additional evidence that copper dependent processes, perhaps induction of *de novo* synthesis of Cu-Zn SOD, have a protective effect with regard to lipid peroxidation [690].

These observations add emphasis to the caution that lipid peroxidation measurements be made on fresh tissue samples [700]. While no phenanthroline-detectable copper, no loss of ceruloplasmin activity, and very little lipid peroxidation were found for samples stored for 4 weeks in the cold, there was a marked increase in phenanthroline-detectable copper, a loss in ceruloplasmin activity, and an increase in lipid peroxidation in these samples after 4 weeks of storage at 40 °C. Storage of these samples at −20 °C or −70 °C slowed but did not prevent these artifactual deteriorations in tissue samples. This may also provide some mechanistic insight into the observation that Cu-Zn-SOD-treated adjuvant arthritic rats experienced less paw swelling and tissue lipid peroxidations ($P < 0.001$) [701].

These observations are also consistent with copper complex modulation of Δ^9 terminal desaturase activity [702]. A purified chicken liver microsomal preparation of stearoyl-CoA Δ^9-desaturase was inhibited with Cu(II)(tyrosinate)$_2$, Cu(II)(lysinate)$_2$ and Cu(II)(histidinate)$_2$. These chelates lowered the steady-state level of ferrocytochrome only 20% and only partially inhibited the NADH-ferricyanide reductase activity, indicating that the terminal desaturation step was being inhibited. Since oxygen is known to be involved in this desaturation and that there is an initial reduction of the desaturase iron, it is plausible that these chelates are decreasing desaturase activity by acting as superoxide scavengers [702].

Increases in *in vivo* lipid peroxidation (pentane exhalation) and serum glutamate pyruvate transaminase (SGPT) activity associated with carbon tetrachloride dosing of rats were decreased 50% with Cu(II)(3,5-DIPS)$_2$ treatment whether the animals were fasted or fed [703]. Mice given acetaminophen and treated with Cu(II)(3,5-DIPS)$_2$ were not studied with regard to the prevention of lipid peroxidation, but treatment with Cu(II)(3,5-DIPS)$_2$ did inhibit the elevation in SGPT activity caused by acetaminophen [703].

Modulation of paraquat toxicity with the mixed-valence complex of penicillamine also involves the prevention of lipid peroxidation [637, 638]. Chloroplast fragments of flax cotyledons had fatty acids that were closer to non-treated control levels than paraquat-treated plants. Decreases in chlorophyll, carotenes and xanthophyll produced by paraquat were also prevented by treatment with the mixed-valence complex. These protective effects of this

complex were considered to be due to its SOD-mimetic effect and it was suggested that the toxicity of paraquat is due to its catalysis of the formation of superoxide [637]. These data also suggested the use of the mixed-valence copper complex of penicillamine to treat human paraquat poisoning.

MODULATION OF ANGIOGENESIS

Folkman and Klagsbrun have recently published an excellent up-to-date overview on angiogenic factors [704]. Angiogenesis is the multiplication and migration of endothelial cells in the formation of new blood vessels, or revascularization of damaged tissue as in wound healing. As Folkman and Klagsbrun point out, there is a recurring theme in the literature indicating that tissue copper modulates the intensity of neovascularization following angiogenic stimulation. Copper complexes formed upon addition of copper to *in vitro* preparations augment endothelial locomotion. Copper complexes increase in the corneal model of angiogenesis before capillaries form in it in response to an angiogenic stimulus such as PGE_1. Ceruloplasmin is angiogenic in the rabbit corneal model, while apoceruloplasmin is not angiogenic. Copper(II)-(heparin) and Cu(II)-(Gly-His-Lys) complexes are also angiogenic in the corneal model, while heparin and Gly-His-Lys are not angiogenic and there is a direct relationship between copper bonding capacity of heparin and its anticoagulant activity. Rabbits fed a copper-deficient diet did not show this angiogenic response until their plasma copper level fell to one-half the normal level.

Folkman and Klagsbrun have hypothesized that solid tumours are dependent upon angiogenesis and that anti-angiogenesis may be an approach to therapy [704]. This has led to the suggestion that copper deficiency might be a therapeutic approach to treatment of cancers [705]. This suggestion would seem to have no merit for at least two reasons. Any suggested inhibition of a normal and vital physiological process as an approach to disease treatment should immediately signal the uselessness of that approach. All serious approaches to disease therapy should attempt to re-establish the normal non-disease state. The second reason is that copper complexes have anti-cancer activity (pp. 505 ff), anticarcinogenic activity (pp. 508 ff) and anti-mutagenic activity (see p. 509 ff), which suggests that the role of copper complexes in angiogenesis should be re-evaluated and at least considered as an antineoplastic response. Neovascularization of neoplastic tumour may be an anti-inflammatory/antineoplastic response which has a role in remission and account for the failure of tumour growth in perfused organs [704].

MODULATION OF LUTEINIZING HORMONE-RELEASING HORMONE SECRETION

Consistent with the newly found role for copper in synthesis of neuroendocrine peptides [18, 19], the copper complex of histidine has been found to increase the secretion of luteinizing hormone-releasing hormone (LH-RH) from granules of the median eminence [705a]. This process was also found to be dependent on chloride transport and consistent with a Cu(II)-histidinate stimulated release of LH-RH via an exocytotic event associated with the influx of chloride which causes release by chemiosmotic lysis [705b]. This secretion was also found to be dependent upon the extracellular sodium concentration, which supported the proposition that Cu(II)-histidinate is taken up by the LH-RH axonal terminals by co-transport with sodium as mediated by Na^+/K^+-ATPase [705c]. Most recently it has been demonstrated that Cu(II)-histidinate amplifies the PGE_2-stimulated release of LH-RH as a postreceptor event involving activation of adenylate cyclase and cAMP plus calcium in the modulation of this release [705d]. All of this evidence supports a key role for copper-dependent processes in normal physiological function of the central nervous system in regulating sex-related physiological events and offers another partial accounting for the high copper content of the brain [705e].

CHEMICAL STUDIES OF BIOLOGICALLY RELEVANT COPPER COMPLEXES

Perrin [706–709], Williams, May and Linder [5, 710–712], and Berthon [300, 301, 713, 714] and their colleagues have developed computer models to study complex formation between low- and high-molecular-weight ligands, such as amino acids, drugs and plasma peptides, and a variety of essential metalloelements, including copper. With their models, they have been able to list possible complexes of copper present in blood, the relative amounts of each, and those responsible for tissue distribution and excretion of copper. Computer modelling has also provided experimental evidence in support of hypotheses that the administration of low-molecular-weight complexes would be beneficial in the treatment of arthritic, ulcer, infections and epileptic diseases [713, 715]. Studies of the physicochemical requirements for bonding of antiarthritic drugs with copper-albumin, a loosely bonded from of copper in human blood, demonstrated copper release and the formation of pharmacoactive forms of these drugs [716]. Studies of copper and albumin bonding

demonstrated that metalloelements are bonded to this peptide and support the generally accepted view that albumin complexes are transportation forms of metalloelements [717]. Results of studies relevant to cupresis in arthritis patients given penicillamine suggest that this drug increases the amount of exchangeable copper in plasma by liberating copper from metalloproteins, which was then instrumental in controlling inflammation [718]. Stabilities of copper complexes of salicylic and acetylsalicylic acids were determined to estimate their concentrations in tissues as they pass through the body. It was concluded that the Cu(II)(salicylate)$_2$ complex was more absorbable and that the complex dissociated at some point in the process of causing anti-inflammatory and antiulcer effects [719, 720]. It was suggested that all effective copper complexes, including copper complexes of antibiotics, were able to pass through membranes and facilitate tissue distribution of copper [713, 719, 720].

Additional studies on the chemical influence of penicillamine on essential metalloelement metabolism demonstrated that 0.15 g/kg body weight given intramuscularly to rats twice daily for 20 days increased urinary excretion of copper by up to 400% [721–724]. Statistically significant reductions of copper were also found for all tissues examined: liver, spleen, kidney, heart, lung, brain and blood (both red blood cells and plasma). In addition, serum ceruloplasmin activity was reduced 69% and cytochrome oxidase activity was reduced 5–17% in these tissues. These studies also demonstrated that the same unspecified weakly bonded pool of copper was the source of copper loss mediated by penicillamine, which was consistent with earlier results [725]. It was also noted that penicillamine inhibited the oxidation of *p*-phenylenediamine by ceruloplasmin [722]. Studies with cysteine revealed that it was rapidly oxidized by ceruloplasmin, presumably to the disulphide. This observation suggests that some of the loss of tissue metalloelements following treatment with penicillamine may be mediated by penicillamine disulphide. Alternatively, penicillamine disulphide formation may account for a loss of the active form of this drug, the parent mercaptan, and may also partially account for the need for large doses in therapy. Since penicillamine therapy of arthritis causes a net loss of copper and other essential metalloelements, penicillamine therapy must be supported with a guaranteed essential metalloelement intake or therapy with a copper complex of penicillamine would be best and consistent with the current understanding of the role this essential metalloelement plays in bringing about disease remission and avoiding toxicities associated with penicillamine therapy [91].

Complex formation and redox reaction of Cu(II) and penicillamine have been studied in detail as a function of metalloelement:ligand ratio and concen-

tration of halide ions using electron spin resonance [726]. A pale yellow polymeric Cu(I) penicillamine complex, Cu(I)(penicillamine), is formed when excess penicillamine is present. The mixed-valence, red-violet complex, $Na_5Cu(I)_8Cu(II)_6(penicillamine)_{12}Cl$, is formed when the ratio of penicillamine to Cu(II) equals 1 and halide ions are present. Complex formation with penicillamine and Cu(II) was also studied in the presence of albumin, alanine, histidine and zinc(II). The mixed-valence complex was again the major species formed at neutral pH. It was suggested that at concentrations found in blood plasma it is unlikely that the mixed valence or the disulphide complexes were not significant contributors to the therapeutic action of penicillamine in the treatment of Wilson's disease. Earlier findings that penicillamine was unable to mobilize copper bonded to albumin [727] were reinforced in these studies [726] and it was pointed out that there was significant non-copper-mediated protein bonding of penicillamine, but this could not be used to account for the depletion of copper in Wilson's disease.

Electron spin resonance studies of the structural features of binuclear copper carboxylates suggest that the magnetic properties of biologically important molecules which contain copper may be better understood with this spectrophotometric technique [728]. Indeed, Greenaway, Norris and Sorenson used electron spin resonance to show that the copper complex of 3,5-diisopropylsalicylic acid is actually isolated as a binuclear complex, $Cu(II)_2(3,5\text{-}DIPS)_4(H_2O)_2$, as opposed to the mononuclear structure, $Cu(II)(3,5\text{-}DIPS)_2$, used throughout this manuscript, and organic solvates of this complex are also binuclear [729]. Electron spin resonance has also been used to study the orientation and mobility of Cu(II)[3-ethoxy-2-oxobutyraldehyde bis(dimethylthiosemicarbazone)] in a bilayer lipid vesicle [730].

Finally, several excellent reviews of inorganic chemical aspects of research dealing with biological systems have been published recently [165, 731–735]. With more of this type of chemical information to hand, it may be possible to develop a better pharmacological approach to the treatment of chronic diseases. It still seems possible that the anti-inflammatory and other actions of analgesics, such as aspirin, and also the anticollagen effect of D-penicillamine, may all be linked by their effect on tissue copper, and that collaboration between workers in metalloelement metabolism and those in experimental therapeutics would prove fruitful [736].

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