PROGRESS IN MEDICINAL CHEMISTRY 15

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Progress in
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Preface

A wide variety of topics in medicinal chemistry is presented in this volume. The chemistry and biological and medical applications of stable isotopes are surveyed in Chapter 1. Cell membranes have an important role in the susceptibility of bacteria to the action of drugs and Chapter 2 reviews recent work in this field. A group of antibiotics which act on the cell nucleus and are therefore of interest as cytotoxic drugs are discussed in Chapter 3.

Our current knowledge of the functional relationships between the hypothalamus and the anterior pituitary gland is discussed in Chapter 4. The chemistry, actions and control of hypophysiotropic hormones are well covered. Much controversy exists over the value of copper complexes in the treatment of rheumatic disorders, although these compounds are active in many animal models of inflammation. Chapter 5 surveys this problem and should stimulate medicinal chemists to attempt the preparation of less toxic and more effective and stable complexes.

Antidepressants other than those of the imipramine type show great chemical diversity and are considered together in Chapter 6. A purely synthetic chemical approach to the treatment of cancer is provided by the thiosemicarbazones which are reviewed in Chapter 7. The final chapter brings up to date a review in Volume 8 of the very rapidly developing field of prostaglandins.

We thank our authors for the considerable efforts they have made in preparing these reviews. We are also grateful to the owners of copyright material for their permission to use it in this volume and to the staff of the publishers for their constant help and co-operation.

November 1977

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Contents

Contents of earlier volumes

VOLUME 7

VOLUME 8

VOLUME 9

VOLUME 10

VOLUME 11

1 STEREOCHEMICAL ASPECTS OF PARASYMPATHOMIMETICS AND THEIR ANTAGONISTS: RECENT DEVELOPMENTS–A.F. Casy
2 QUANTUM CHEMISTRY IN DRUG RESEARCH–W.G. Richards and M.E. Black
3 PSYCHOTOMIMETICS OF THE CONVOLVULACEAE–R.A. Heacock
4 ANTIHYPERLIPIDAEMIC AGENTS–E.-C. Witte
5 THE MEDICINAL CHEMISTRY OF LITHIUM–E. Bailey, P.A. Bond, B.A. Brooks, M. Dimitrakoudi, F.D. Jenner, A. Judd, C.R. Lee, E.A. Lenton, S. McNeil, R.J. Pollitt, G.A. Sampson and E.A. Thompson

VOLUME 12

1 GAS–LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY IN BIOCHEMISTRY, PHARMACOLOGY AND TOXICOLOGY–A.M. Lawson and G.H. Draffan
2 RECENT ADVANCES IN COLUMN CHROMATOGRAPHY–K.W. Williams and R.C. Smith
3 NMR SPECTROSCOPY IN BIOLOGICAL SCIENCES–P.J. Sadler
4 ELECTRON SPIN RESONANCE IN MEDICINAL CHEMISTRY–D.L. Williams-Smith and S.J. Wyard
5 POLAROGRAPHY IN BIOCHEMISTRY, PHARMACOLOGY AND TOXICOLOGY–M. Březina and J. Volke
6 METHODS RELATED TO CYCLIC AMP AND ADENYLATE CYCLASE–B.G. Benfey
7 RESISTANCE OF *PSEUDOMONAS AERUGINOSA* TO ANTIMICROBIAL DRUGS–R.B. Sykes and A. Morris
8 FUNCTIONAL MODIFICATIONS AND NUCLEAR ANALOGUES OF β-LACTAM ANTIBIOTICS–Part I–J.Cs. Jászberényi and T.E. Gunda

VOLUME 13

1 CLINICAL ENZYMOLOGY–David M. Goldberg
2 THE RELEASE OF PHARMACOLOGICALLY ACTIVE SUBSTANCES IN PARASITIC INFECTIONS–P.F.L. Boreham and I.G. Wright
3 THE MEDICINAL CHEMISTRY OF 1,2,3-TRIAZINES–Malcolm F.G. Stevens
4 THE BIOLOGICAL USES AND IMPORTANCE OF GLUTARALDEHYDE–A.D. Russell and D. Hopwood
5 THE CHEMISTRY AND BIOCHEMISTRY OF C-NUCLEOSIDES–G. Doyle Daves, Jr. and C.C. Cheng

VOLUME 14

1 ASPECTS OF THE PHARMACOLOGY OF PASSIVE ION TRANSFER ACROSS CELL MEMBRANES–A.W. Cuthbert
2 THE BIO-MEDICAL AND RELATED ROLES OF ION-SELECTIVE MEMBRANE ELECTRODES–G.J. Moody and J.D.R. Thomas
3 BIOLOGICAL ACTIVITY OF POLYENE ANTIBIOTICS–S.M. Hammond
4 FUNCTIONAL MODIFICATIONS AND NUCLEAR ANALOGUES OF β-LACTAM ANTIBIOTICS–PART II–E.T. Gunda and J.Cs.Jászberényi
5 THE ROLE OF BIOGENIC AGENTS IN THE ACTIONS OF CENTRALLY-ACTING ANALGESICS–R.D.E. Sewell and P.S.J. Spencer
6 FLAVONOIDS: SOME PHYSIOLOGICAL AND NUTRITIONAL CONSIDERATIONS–R.E. Hughes and H.K. Wilson

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1 The Use of Stable Isotopes in Medicinal Chemistry

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INTRODUCTION

The intention in this review is to illustrate the use of stable isotopes in fields of interest to the medicinal chemist. Selection is inevitable. It is therefore proposed to outline the more important methods used for the separation of the isotopes and to describe some of the problems that confront the synthetic chemist in the preparation of labelled compounds. Of the wide variety of applications, the potential in clinical work has as yet been barely realised and it is therefore opportune to pay particular attention to such uses. The isotope mass spectrometer is of especial importance in such studies and this technique will be treated in some detail.

Other important areas of concern to the medical chemist include pharmacology, biochemistry, and the study of biosynthetic pathways. These will be illustrated and reference made to a number of reviews that are available. Equally,

other assay and detection methods including nuclear magnetic resonance (NMR), optical emission, and infrared spectrometry will be described in less detail. However, this should in no way be taken as minimising their importance in particular isotope studies.

The heavy stable isotopes of carbon (^{13}C), nitrogen (^{15}N), and oxygen (^{17}O and ^{18}O), and the widely used deuterium, were all discovered in the period 1929–1932 and they soon assumed an important role in biochemical studies. A wealth of material on the separation and detection of stable isotopes, the synthesis of labelled compounds, their use in biochemistry, and especially for the study of the metabolism of proteins, carbohydrates, and lipids, was reported at a symposium in 1947 [1]. The subsequent widespread use of radioactive isotopes in a variety of disciplines of interest to the medicinal chemist eclipsed the use of their stable counterparts which were at a disadvantage both on grounds of isotope cost and lack of instrumentation. However, since the late 1960s, there has been a rapid change in the situation. Developments in instrumentation such as mass spectrometry, especially when linked with the gas chromatograph, and Fourier transform NMR, have opened up immense opportunities. Large reductions occurred in the price of the more commonly used stable isotopes, and since that time, prices have remained steady or fallen slightly. The resurgence of the use of stable isotopes in the life sciences has been underlined at international symposia that have been devoted to work in the area [2–7]; much important work is available in the published proceedings.

In many circumstances, stable isotopes offer advantages over their radioactive counterparts. They do not have associated problems of radiation, while the synthesis and handling of labelled compounds, which do not suffer from inherent problems of stability, is relatively simple. The lack of radioactivity is of particular importance in clinical studies, especially, but not only, in the case of children and women of child-bearing age. Where pharmacological studies involve large body pools and low plasma levels for measurement, the use of stable isotope labelled compounds is essential. With nitrogen and oxygen, there are no competing radioisotopes with a long enough half-life to have adequate utility as labels. Substances of interest in metabolic and biosynthetic studies may not only be non-specifically detected, as is the case with radioisotopes, but accurate assessments of the position of the labelled atom in the particular molecule can normally be made, especially when using techniques such as ^{13}C-NMR. Furthermore, in certain circumstances, the specific detection method can be comparable in sensitivity with that of non-specific detection methods used for radioisotopes [8]. The complementary use of both radio and stable isotopes can be a potent tool for the solution of many problems, especially in biosynthetic studies.

The medicinal chemist is primarily concerned with organic compounds and the stable isotopes of most interest are deuterium, carbon-13, nitrogen-15, and

oxygen-17 and -18. There is a very extensive literature on the use of deuterium and reviews are available [9–13]. In much of this review, discussion will be essentially confined to the isotopes of carbon, nitrogen, and oxygen, except that in the case of quantitative clinical studies, the important role of deuterium will be additionally described.

Since the stable isotopes that will be discussed are naturally occurring (the natural abundances are ^{2}H, 0.015; ^{13}C, 1.1; ^{15}N, 0.36; ^{17}O, 0.04; ^{18}O, 0.2%), they are not in themselves alien to animal systems. However, questions of toxicity inevitably arise. Although there are no radiation hazards associated with the isotopes themselves, the commonly available deuterium compounds may contain a significant amount of tritium which is simultaneously enriched in the separation process. Carbon-14 does not currently present an analogous problem since most of the world supplies of carbon-13 use a geological source of carbon as feedstock for their separation processes. In the case of nitrogen and oxygen, with only very short-lived radioisotopes, the problem does not arise.

Any harmful effects would derive from the difference in mass and many instances have been recorded of isotope effects in biological systems. There is an inherent probability that these effects would be most pronounced in the case of deuterium where there is a 2-fold increase in mass and many instances have been reviewed [9,12,14]. Carbon-13 isotope effects have been reported in biological systems and algae and yeast have both been grown with ^{13}C labelling at above 90 atom% with no apparent adverse effects [15]. More dramatically, 2 mice were kept on a diet of yeast, one of them for 6 months, with about 90 atom% ^{13}C [16]. The mice ultimately had a body carbon content of about 60% ^{13}C but no gross anatomical or histological abnormalities were detected on post-mortem examination. Studies have also been made with a view to detecting teratological effects in mouse embryos [17–19]; no adverse effects have been detected with ^{13}C incorporation up to 20%. These aspects of the use of stable isotopes must be kept constantly under review, but to date, biological effects of the heavy isotopes of carbon, nitrogen, and oxygen do not detract from their usefulness. However, in recent studies on the metabolism of phenazone (antipyrine), caffeine, and pethidine (meperidine), switching of the metabolic pathways occurred on substitution of deuterium in certain positions in the molecule [20,21]. This was an isotope effect and, although possible, it is unlikely that it would be so pronounced with carbon-13 where the atomic mass difference is relatively so much smaller.

In recent years, the lighter isotopes have assumed more significance and importance. By way of nomenclature, throughout this review, carbon-12 refers to carbon depleted of carbon-13, nitrogen-14 to nitrogen depleted of nitrogen-15 and so on. Natural material is referred to as carbon, nitrogen and so on.

AVAILABILITY OF STABLE ISOTOPES

The stable isotopes of carbon, nitrogen, oxygen, and sulphur are available commercially in a variety of chemical forms. In this section, an outline is given of the various methods that are currently used for the separation of stable isotopes. It will be clear that the primary source of isotope, and therefore, in most cases the cheapest available form, is determined by the separation procedure adopted. A discussion of some aspects of the synthesis of labelled compounds follows. Although in a review of this size, this cannot be comprehensive, the methods used and the problems that are raised will be outlined. Some recent developments that increase the choice of chemical form available will be mentioned.

Isotope content is expressed as atom%, defined as the ratio of the number of atoms of the isotope to the total number of atoms of that element expressed as a percentage. It only refers to the atoms in the labelled position(s). For example, in sodium [1-^{13}C]acetate at 90 atom%, 90% of the carbonyl carbons are ^{13}C, while ^{13}C occurs only to the extent of the natural abundance in the methyl group. Mole% is sometimes used where atoms are replaced by moles in the above ratio. Isotopic enrichment is an expression that is frequently loosely used. Strictly speaking it indicates the change in atom% that has occurred in a separation process and most commonly represents the increase in abundance above the natural level; it is expressed as atom% excess.

SEPARATION OF STABLE ISOTOPES

This review is confined to the separation of the isotopes of carbon, nitrogen, oxygen, and sulphur. Although the natural abundance of these isotopes is low, the substitution of an isotope in a molecule produces changes, albeit small, in both the chemical and physical properties. It is these changes that have been utilised in the different methods that have been adopted and successfully exploited for the separation of isotopes on the commercial scale. The basic principles have been well documented [22] and the present commercial processes have been reviewed [23].

The fractionation of two isotopes (A and A′) in equilibrium between two phases (I and II) is determined by the equilibrium constant of the reversible process illustrated in Equation (1).

$$\underset{\text{phase I}}{AX} + \underset{\text{phase II}}{A'Y} \rightleftharpoons \underset{\text{phase I}}{A'X} + \underset{\text{phase II}}{AY} \qquad (1)$$

The constant, known as the separation factor (α), may be represented by the

expression:

$$\alpha = \frac{A'/A_I}{A'/A_{II}}$$

Because of the small difference in the properties of the isotopic species, separation factors are close to unity and to achieve appreciable fractionation, a large number of separation stages must be incorporated in the plant. Thus, distillations, exchange reactions, and thermal diffusions are carried out in long fractionation columns or in cascades of several fractionating units in order to achieve the maximum separation. To ensure efficient operation, the packing of the columns is a critical factor in their construction. Continuous operation is essential and so conditions in a given plant are critical; automated controls and alarm systems must be incorporated. The building of an isotope separation plant clearly requires a variety of specialised skills and is therefore normally the concern of specialist organisations. The following brief outline of the separation of the heavy isotopes of carbon, nitrogen, oxygen, and sulphur illustrates the application of the more important methods.

Carbon isotopes

Most of the carbon-13 used is separated using the cryogenic distillation of carbon monoxide. The separation factor at the operating temperature is 1.008. This method is adopted at the ERDA facility at Los Alamos, USA [24] and by Prochem in London, England. Although claims have been made that carbon-13 can be produced more cheaply by chemical exchange [25], this has yet to be proved commercially. The reliability of the system and the simplicity of the technology ensures that the distillation of carbon monoxide retains its dominant role for the separation of carbon-13 at 90–93 atom% for some time to come.

A schematic illustration of the type of fractionating column used at Prochem is shown in *Figure 1.* Three random packed columns are mounted in a common vacuum jacket sunk in a 20 m bore hole and served by a common condenser. Two of the columns are identical and stepped as shown, ranging from 6.5 cm in diameter at the condenser end to 2.5 cm at the boiler. Operating on a fixed time cycle, carbon monoxide is enriched to about 12 atom% and fed to the third column, a high efficiency second stage unit of 2.5 cm diameter throughout its length, which lifts the enrichment above 90 atom%. The process is fully automated and as no bulky ancilliaries are required at the boiler end, a compact unit has been constructed and sunk in a rigid bore hole [26]. A similar method has been adopted at Los Alamos to accommodate their very long units although their column arrangement is somewhat different [24].

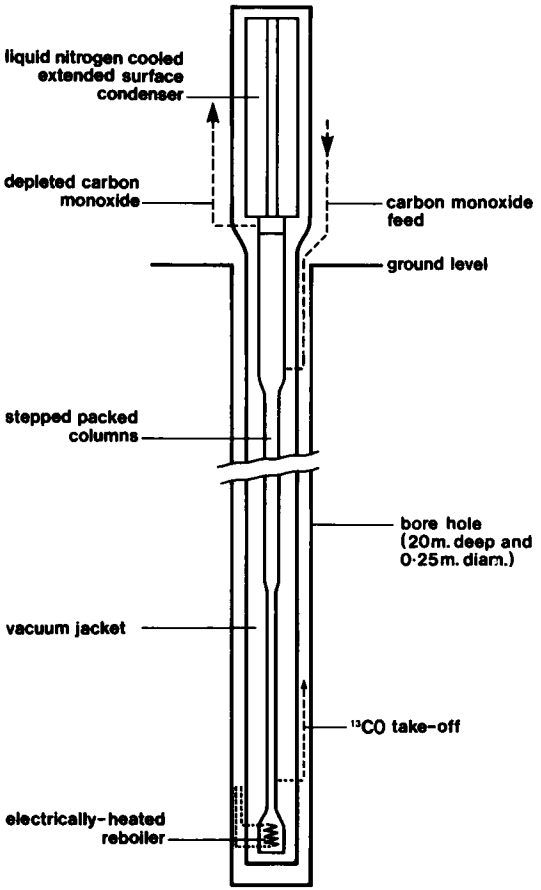


Figure 1.1. Schematic illustration of primary column for the separation of carbon-13 (Prochem, London)

Carbon-12 is readily produced either by rearranging the operation of the column or by feeding the waste-gas to a similar unit for further fractionation giving a product of 99.95 atom% ^{12}C.

Higher enrichment carbon-13 is rather more problematical. Cryogenic distillation of carbon monoxide was believed to be limited in its enrichment capability by the difficulty of separating $^{13}C^{16}O$ and $^{12}C^{18}O$. This does not appear to be insuperable and by transfer of product to a second unit, carbon monoxide at

98 atom% has been produced at Prochem and further improvements are envisaged [27]. Thermal diffusion of methane [28,29] will separate carbon-13 at 99 atom% and plants of this type have been operated by Mound Laboratories in the USA. In general, thermal diffusion uses columns in the form of an outer water-cooled tube with an axial heating element. The lighter isotope normally diffuses up the temperature gradient and a separation can be achieved. With a 24.3 m column and a hot wire as the heating element, methane was separated at 99.8 atom% ^{13}C as early as 1954 [28].

Chemical exchange methods for the separation of isotopes utilise the small differences of concentration that exist at equilibrium in a reversible reaction involving two chemical substances. As a method of enriching carbon-13, it was discovered that the formation of salt-type carbamates (2) when carbon dioxide combines with amines (1) in non-aqueous solutions (Equation 2) fulfilled the desired conditions [30–32]. Oxygen-18 enrichment occurred in the gaseous carbon dioxide and carbon-13 in the carbamate (2). The enriched carbamate readily

$$\underset{(1)}{2\,R_2NH} + CO_2 \rightleftharpoons \underset{(2)}{(R_2NCO_2)^-(H_2NR_2)^+} \qquad (2)$$

decomposed to its components on heating. The procedure adopted at the Mound Laboratories used a solution of di-*n*-butylamine (1, R = Bu) in triethylamine solution. The process can be carried out at ambient temperatures with a separation factor of 1.01 for ^{13}C.

The so-called Cyanex system [25] is a liquid–liquid chemical exchange process based on the reaction shown in Equation 3, the exchange occurring on partition between an organic solvent, such as xylene and water. The carbon-13 con-

$$Et_2C(OH)^{12}CN_{(org)} + K^{13}CN_{(aq)} \rightleftharpoons Et_2C(OH)^{13}CN_{(org)} + K^{12}CN_{(aq)} \qquad (3)$$

centrates in the cyanohydrin species. Separation factors of 1.04 were achievable and were claimed to be the highest reported for carbon isotope fractionation.

Other systems for enrichment of carbon-13 have utilised the exchange between hydrogen cyanide gas and aqueous solutions of sodium cyanide [33, 34], and exchange between carbon dioxide and aqueous solutions of sodium bicarbonate [34,35].

Nitrogen isotopes

There are a number of methods that have been used for the separation of nitrogen-15 and these have been reviewed [36]. The two most important, that

now have a well-established place in commercial production, utilise the cryogenic distillation of nitric oxide [37–39] and chemical exchange between oxides of nitrogen and nitric acid [40–43].

In the low temperature distillation of nitric oxide for the separation of the 2 isotopes of nitrogen and 3 of oxygen, a 6 component system must be considered so far as isotopic separation is concerned. Whilst the basic principles are the same as in the cryogenic distillation of carbon monoxide, the practical process is of necessity more complex. Extensive purification of the feed gas is essential and the potential explosion hazard of liquid nitric oxide poses special handling problems. Furthermore, the freezing point of nitric oxide (−163°C) does not allow liquid nitrogen to be used as a direct coolant. Some aspects of the US ERDA plant at Los Alamos have been reviewed [23,39]. The second method, the so-called Nitrox process, is used at a number of centres in Europe, including Cluj (Romania) [36], Leipzig (GDR) [44], and London (England) [26]. The optimum separation factor is 1.055 and a highly efficient and reliable system can be constructed to produce significant quantities of nitrogen-15 assaying at up to 99.9 atom% ^{15}N [26] with only minimal needs for supervision. However, the separation of the enriched oxygen-18 is not practicable and as yet, it does not appear to have been used for the production of nitrogen-14. The most serious disadvantage of the process is the highly corrosive nature of the refluxing gases and acids.

A diagrammatic illustration of a Nitrox plant is shown in *Figure 1.2*. Oxides of nitrogen are liberated from the nitric acid by the action of sulphur dioxide in the bottom refluxer, the sulphuric acid formed going to waste. Exchange takes place in the separating column according to Equation 4. The rising gas is reconverted

$$\underset{\text{(gas)}}{^{15}NO} + \underset{\text{(liq)}}{H^{14}NO_3} \rightleftharpoons \underset{\text{(gas)}}{^{14}NO} + \underset{\text{(liq)}}{H^{15}NO_3} \qquad (4)$$

to the liquid phase by addition of water and oxygen in the top refluxer. The Prochem plant uses a three stage system of packed columns, 7 m high and varying in diameter from 7.5 to 2.2 cm, and linked with suitable metering pumps capable of handling the corrosive gas saturated acids in the system [26].

Other chemical exchange reactions that have been used for the fractionation of nitrogen isotopes include ammonia and ammonium nitrate solution [45], ammonia and ammonium carbonate [46], and ammonia and its complexes with aliphatic alcohols [47].

Thermal diffusion of nitrogen afforded 98.95% of the $^{14}N^{15}N$ species [48]. It has also been used to prepare nitrogen at 99.8 atom% ^{15}N [49], the $^{14}N^{15}N$ molecules being randomised by the action of an electric discharge. Other work has been reported using chemical exchange of nitric oxide and nitrogen dioxide in a thermal diffusion column [50].

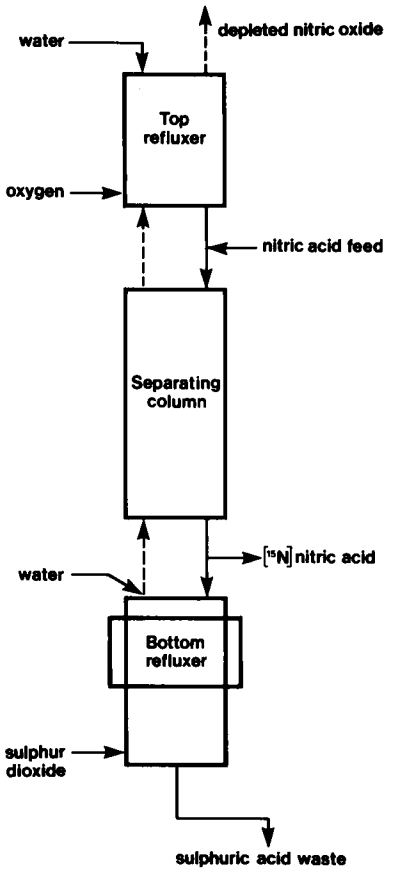


Figure 1.2. Schematic illustration of the Nitrox process for the separation of nitrogen-15

Nitrogen isotopes have been separated by gas chromatography. The separation of $^{14}N_2$ from $^{15}N_2$ was effected on a graphon column at 77°K [51], a partial separation having been previously obtained on glass capillary columns [52]. A laser-induced dissociation reaction of *s*-tetrazine has been reported to show separation of nitrogen-14 and -15, and of carbon-12 and -13 [53].

Oxygen isotopes

Oxygen-18 was first produced commercially at the Weizmann Institute in Israel from the distillation of water [54] and this method has subsequently been

developed in combination with thermal diffusion to have a capability of producing oxygen-18 at 99.9 atom% and oxygen-17 at 96 atom% [55].

At the Nuclear Research Centre at Karlsruhe (FRG) oxygen-17 and -18 are separated by distillation of heavy water. This work, which is carried out in collaboration with Norsk–Hydro, capitalises on the fact that there is enrichment of the heavy oxygen isotopes in the Norwegian manufacture of heavy water [23]. In this plant, the features of which have been described [56], oxygen-18 is produced at 99.9 atom%. Intermediate product is converted into H_2O and fed to a distillation unit in order to produce useful enrichments of oxygen-17 (approaching 30 atom%). Water-^{16}O (depleted in both ^{17}O and ^{18}O) is produced at 99.99 atom% ^{16}O [56].

The separation of oxygen-16, -17 and -18 by cryogenic distillation of nitric oxide is used at Los Alamos (USA) and has been referred to in the previous section on the separation of nitrogen isotopes.

Cryogenic distillation of oxygen is an effective method of producing oxygen-18 economically at lower enrichments (25 atom%). The method is limited by the fact that oxygen-18 in the feedstock is present as $^{16}O^{18}O$. By randomisation of the molecular species, for example by heating, higher enrichments could be attained [26]. The technique provides a ready source of depleted material ($^{16}O_2$).

Sulphur isotopes

Although little work has been reported on the use of sulphur isotopes in areas of interest to the medicinal chemist, sulphur-34 could well play a significant role in the future. Some comments, therefore, are not out of place.

Sulphur-34 provides an example of the use of thermal diffusion in the liquid phase. This separation has been effected at the Mound Laboratory (USA) using carbon disulphide in concentric columns. The separation takes place in the annulus between a steam heated inner tube and a water cooled outer one [57].

However, the currently preferred method, also on-stream at Mound Laboratory, as well as at CEA in France, utilises the exchange reaction between sulphur dioxide and sodium bisulphite (Equation 5). The single stage separation factor is 1.011 [57,58].

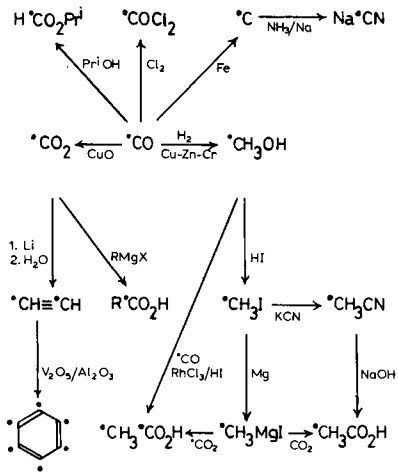
$$\underset{\text{(gas)}}{^{34}SO_2} + \underset{\text{(liq)}}{H^{32}SO_3^-} \rightleftharpoons \underset{\text{(gas)}}{^{32}SO_2} + \underset{\text{(liq)}}{H^{34}SO_3^-} \qquad (5)$$

SYNTHESIS OF LABELLED COMPOUNDS

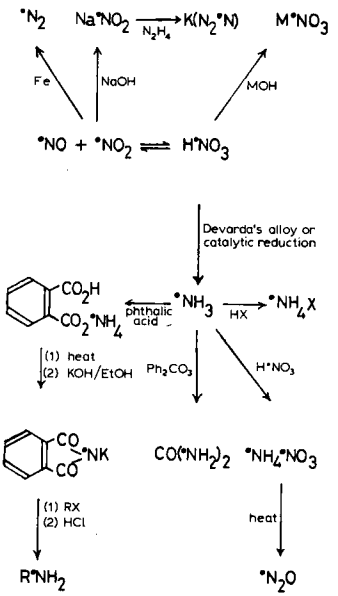
To the medicinal chemist using stable isotopes, the first problem is the source and choice of a particular compound for the required studies. From the previous part of this section, it will be clear that the form of the separated isotope does not always conform to expectations as the ideal synthetic starting point. It is proposed to give some guidelines on the synthesis of labelled compounds from the raw isotope and to enunciate principles designed to aid the chemist in the most efficient use of his resources.

The manufacturers who separate stable isotopes, and a number of other suppliers, offer a range of labelled compounds. These consist of widely used important synthetic intermediates, together with a range of rather more specialised compounds.

Synthetic problems arise principally from the nature of the starting material and the price of the isotope. Isotope prices have been fairly stable or fallen slightly over the last five years; as this has coincided with inflation, in real terms they are probably now at a historical low; the basic cost of deuterium as heavy water is now in fact markedly rising. Chemical synthesis is frequently labour intensive and therefore on the small scale, isotope price is not a significant part of the cost of labelled compounds; with isotope separation procedures having reached a high degree of efficiency, only increased consumption leading to large-



Scheme 1.1. The preparation of some key intermediates labelled with carbon-13. (Labelled carbon atoms are indicated by •)



Scheme 1.2. The preparation of some key intermediates labelled with nitrogen-15. (Labelled nitrogen atoms are indicated by •)

scale syntheses with concomitant savings on cost is likely to effectively reduce their price.

The syntheses of some ^{13}C-labelled key intermediates from ^{13}CO are illustrated in *Scheme 1.1.* These compounds are prepared on a relatively large-scale by manufacturers. Yields are high; as the methods are frequently scaled-down versions of industrial processes or utilise specialised equipment, they are not always suitable for use in the majority of chemical laboratories. For example, [^{13}C]methanol is prepared by catalytic reduction of ^{13}CO with hydrogen under pressure [59]. The method can be automated and used for the continuous preparation of large batches. In the majority of cases, the user may well find that he is unable to prepare intermediates of this type as cheaply as they can be purchased. *Scheme 2.2* illustrates the preparation of widely used ^{15}N-labelled compounds. More detailed reviews of the basic synthetic processes have been published [15, 26,60–63] and many of the preparations have been described in individual publications. Some more specialised labelled compounds such as amino acids, fatty acids, organic heterocycles, and sugars are also available commercially.

The medicinal chemist wishing to use compounds labelled with stable isotopes will need to consider the following aspects when considering his synthetic procedures.

(a) The position or positions to be labelled in the molecule. This may be dictated by the possible synthetic routes and the detection methods that are to be employed.

(b) How many atoms in the molecule should be labelled, and whether the labelling in a multilabelled compound should be homogeneous or heterogeneous.

(c) The yields expected and hence the economic viability of the process. If cost of the isotope is an important factor, chemical yields should be high or the possibility of recovery of unused isotope should be considered

(d) The metabolism of the compound in biological studies to determine the most suitable position(s) for labelling.

(e) Whether there is any likelihood of isotopic exchange. Oxygen-17 and -18 labelled compounds are frequently prepared by exchange reactions. The possibility of exchange occurring either in subsequent steps in the synthesis or in the process under study must be considered. Such problems are inherently less likely with the isotopes of carbon and nitrogen. However, the possibility must not be overlooked. For example, in some 2-aminopyridines, it has been shown that exchange occurs between the nitrogen atoms under mild hydrolytic conditions [64].

(f) The isotopic enrichment required. This may be related to (i) the sensitivity of the detection methods used, (ii) isotopic dilution in the system being studied, and (iii) where multiple labelled compounds are being employed, the absolute amount of the multiple labelled species itself that is present. For example, sodium [1,2-^{13}C]acetate at 90 atom% contains about 81% of double labelled molecules, about 8.5% each of the single labelled species, and some 2% of unlabelled compound. At 95 atom%, the proportion of double labelled species rises to over 90%.

Within the constraints referred to above, the synthesis of compounds labelled with stable isotopes follows the standard practices of synthetic chemistry. Unlike radioactive labelled compounds, the syntheses present no radiation hazard nor any special problems of stability. Subsequent sections of this chapter will illustrate the wide range of labelled compounds that have been prepared in order to solve particular problems.

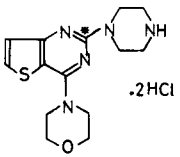
Multiple labelled compounds

Multiple labelling of organic compounds is an area that offers a wealth of unrealised potential. Multiple labelling with deuterium has frequently been used in studies of drug metabolism in order to give shifts of several mass numbers on

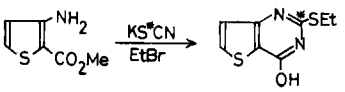
examination of the metabolic products by mass spectrometry. However, as has already been mentioned, metabolic switching of pathways can occur as a consequence of deuterium substitution [20,21]. Results must therefore be evaluated very critically and treated with caution. Although possible, such switching is inherently unlikely with carbon-13 or nitrogen-15. These isotopes should, therefore, ideally be the label of choice. Organic mass spectrometric analysis of a complex mixture, is frequently complicated by the natural abundance of the heavy isotopes. A shift of more than one mass number is therefore highly desirable in order to increase sensitivity and ease interpretation [65,66]. The incredible sensitivity that can be attained by the use of such compounds has been clearly demonstrated with the use of heavy methane ($^{13}CD_4$) as an atmospheric tracer [63,67]. Mass-21 methane is almost non-existent in the atmosphere and has been detected at concentrations ranging from 2 to 10×10^{-17} parts by volume.

Most labelled compounds are synthesized from simple starting materials. Double or triple labelling, either with a homogeneous or heterogeneous combination of heavy isotopes, therefore presents few problems. In most cases, the cost-benefit of the use of such compounds is very favourable, but the enrichment of the isotopes used should be carefully considered since the concentration of the fully labelled species, as compared with the partially labelled form, falls off rapidly as the level of enrichment is reduced.

The use of depleted materials in multiple labelling should not be overlooked but to date, the only example of importance is the use of carbon-12 and deuterium labelled solvents in ^{13}C-NMR spectroscopy. This is an effective method of eliminating interference from the natural abundance ^{13}C of the solvent used, especially when only small samples of material are available.



(3)



(4) (5)

Multiple labelling with both stable and radioactive isotopes offers the advantage of simultaneous labelling in the same position of a molecule [68]. The combination of ^{13}C- and ^{14}C-labelling combines ease of detection with rapid structural identification. The adoption of this technique in studies of some aspects of the metabolism of 4-morpholino-2-piperazinothieno[3,2-*d*]pyrimidine (V-K 774) (3) in the rat has been reported [69]. Potassium [^{13}C,^{14}C]thiocyanate was prepared from a mixture of potassium [^{13}C,^{14}C]cyanides. Reaction with ethyl bromide and methyl 3-aminothiophen-2-carboxylate (4) afforded the thienopyrimidine (5), which, by a series of reactions was converted to V-K 774 (3) labelled in the position shown.

With a number of isotopes readily available commercially, and with methods of synthesis that have reached a high degree of versatility, there is now an enormous potential for the exploration of the application of multiple labelled compounds. This has been discussed on a number of occasions [26,27,63].

Preparation of labelled compounds by biosynthesis

Biosynthetic methods are of importance in the preparation of natural products and related compounds [60]. The requirements of such methods are that a system must be devised that not only produces the desired product, but produces it in a readily isolable form.

The use of a biosynthetic method for the production of a uniformly labelled natural product is well illustrated by the large-scale photosynthetic production of labelled sugars [70]. Tobacco leaves were incubated with $^{13}CO_2$ for a period of 40 h in specially designed chambers. Uniformly labelled starch, glucose, fructose, and sucrose were isolated from the leaves. Uniformly labelled ^{13}C-labelled amino acids have been prepared on the large-scale by biosynthesis using algae, *Spirulina maxima*, grown in the presence of sodium [^{13}C]bicarbonate [71]. Labelled amino acids and other isotopically labelled natural products could well be produced by the culture of other microorganisms, algae, or bacteria [72].

More specific labelling may be obtained in a biosynthetic process using enzymic methods of synthesis. The preparation of amino acids by the reductive amination of 2-keto acids is well known. Indeed, the synthesis of ^{15}N-labelled amino acids by this route is among the earliest literature on the preparation of compounds labelled with stable isotopes [73]. Such reductions can be carried out enzymically and L-[α-^{15}N]glutamic acid has been prepared in good yield from 2-oxoglutaric acid and ^{15}N-labelled ammonium chloride in the presence of reduced nicotinamide adenine dinucleotide phosphate [74].

As will be clear from a subsequent section, a number of antibiotics labelled with stable isotopes have been prepared in studies of their biosynthesis from labelled precursors.

Gas mixtures

The availability of gas mixtures with an isotopically labelled component is a new development *, hitherto little used, but offering further opportunities for the application of stable isotopes. The importance of standardised gas mixtures, some of them very complex, is now well established in a variety of areas. Numerous advantages can be achieved by incorporating a component labelled with a stable isotope [27]. The change in mass number can increase the versatility of analytical techniques since, for example, natural nitrogen and ^{13}CO are clearly distinguished in the mass spectrometer. More meaningful tracer experiments can be conducted in biological systems; synthetic air can be produced with the oxygen, nitrogen, and/or carbon dioxide labelled. Gas mixtures can be a relatively cheap source of stable isotopes. High enrichment nitrogen (essentially mass 30) blended with helium may well be more useful than an equivalent volume of low enrichment nitrogen which will contain large amounts of masses 28 and 29 and relatively little mass 30. Isotopically labelled components can confer desirable properties on a mixture for a specific use. For example, a mixture of ^{13}CO (90 atom%), 3% xenon, 18% nitrogen with balance helium is proving particularly useful in laser work [75].

ANALYTICAL TECHNIQUES

Methods of detection and assay of stable isotopes depend upon the differences in their physical properties. The four most important techniques are mass spectrometry, nuclear magnetic resonance spectrometry, optical emission spectroscopy, and infrared spectroscopy.

Mass spectrometry is the most versatile and widely used technique and is capable of great sensitivity. It can be employed for a whole range of isotope analyses. The analysis of simple gases using the isotope mass spectrometer is of particular importance in clinical applications and will be treated in some detail. The pharmacologist is frequently concerned with complex mixtures and it is in this area that the organic mass spectrometer linked to the gas chromatograph has assumed an important role.

The nuclear magnetic resonance spectrum gives very specific structural information that enables the position of labelled nuclei in a molecule to be defined. The technique has been especially important in studies of biosynthesis. Rapid

* Available from BOC Limited, Prochem, London to customers' requirements.

developments in ^{13}C-NMR have inevitably meant that the most important applications are in this area. However, an increasing amount of work is being carried out with nitrogen-15, and, as more sophisticated instrumentation becomes available, with oxygen-17.

Optical emission spectroscopy is more limited in its application but has nevertheless established an important role. It has found particular use for the measurement of nitrogen-15 levels in agricultural studies. Its limitations arise from the necessity to convert the sample to nitrogen gas for measurement.

In certain cases, infrared spectra give useful and important information on isotope labelling although the method is not of widespread or general importance. Activation analysis and the use of Raman scattering have also been employed for isotope ratio measurements.

MASS SPECTROMETRY

The isotope mass spectrometer

Isotope mass spectrometers are designed to measure the abundance of an isotope in an unknown gas sample compared with that in a known reference gas. The layout of a typical instrument analyser is shown in *Figure 1.3.* There are important differences between the isotope mass spectrometer and the high resolution analytical mass spectrometer. In both types of instrument the sample is ionised

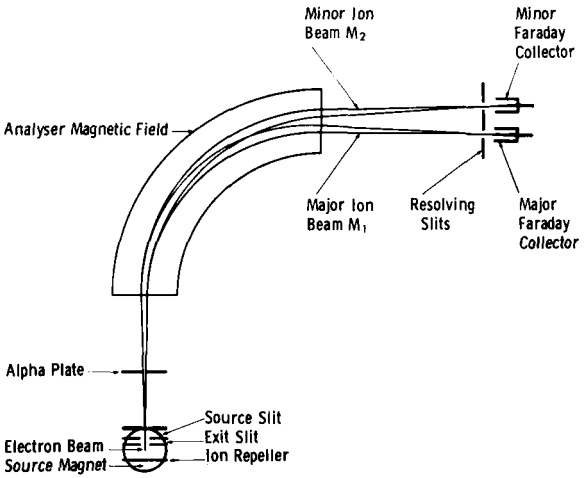


Figure 1.3. Schematic diagram of the analyser tube of an isotope mass spectrometer showing the relative positions of the source and collector assemblies

and propelled down a flight tube which passes between the poles of a magnet. In both instruments the ions are deflected by the magnetic field in proportion to their mass:energy ratio. However, in the high resolution instrument the ion beam is swept over a slit by varying either the strength of the magnetic field or the accelerating voltage so a mass: energy spectrum of the sample can be recorded, whereas in the isotope ratio instrument conditions are held absolutely constant throughout the analysis, and one pair of isotopes only is measured alternately in the unknown and reference gas.

If an ion of mass m and charge e is propelled by an accelerating voltage V through a magnetic field of strength B, it will follow a curved path of radius R. The relationship of these variables is given by:

$$\frac{m}{e} = \frac{B^2R^2}{2V}$$

Thus in *Figure 1.3* if the mass spectrometer is being used to measure the ratio of $^{14:14}N$ to $^{15:14}N$ in a gas sample, the accelerating voltage on the ion repeller plate and the strength of the magnetic field would be chosen so that the $^{14:14}N$ ion with a mass:energy ratio of 28 would follow the curve labelled 'major ion beam M_1', and the heavier $^{15:14}N$ ion would curve less sharply to follow the path of 'minor ion beam M_2'. Each ion beam passes through a resolting slit and delivers its charge to the appropriate collector. The resulting ion current, which is amplified, is proportional to the number of ions impinging on the collector in unit time, and hence to the partial pressure of that isotope species in the gas sample. If a reference gas of known isotope abundance is now introduced into the ion source under identical conditions, the relative abundance of the ^{15}N isotope in the unknown sample can be calculated.

Isotope mass spectrometers typically cover the range $m : e$ 2–100. A high vacuum pumping system is required to maintain a sample inlet pressure of 10^{-4} torr and an analyser pressure of 10^{-9} torr. Both inlet and analyser components can be baked to 300°C to reduce the background spectrum.

The gas inlet system. A schematic diagram of a typical inlet system is shown in *Figure 1.4.* The reference and sample gases are admitted into the inlet system which consists of independently variable metal bellows that are controlled to provide identical major ion currents from both standard and sample gas during analysis. The gases flow through crimped metal capillary leaks to a glass or metal changeover valve [76] so that the reference gas enters the analyser source, and the sample gas is pumped away through a 'bleed' system preserving the gas flow throughout the analysis. The solenoid operated changeover valve facilitates direct comparison of the isotopic content of reference and sample gases on a strict time basis. Balanced flow through the inlet system is rapidly achieved

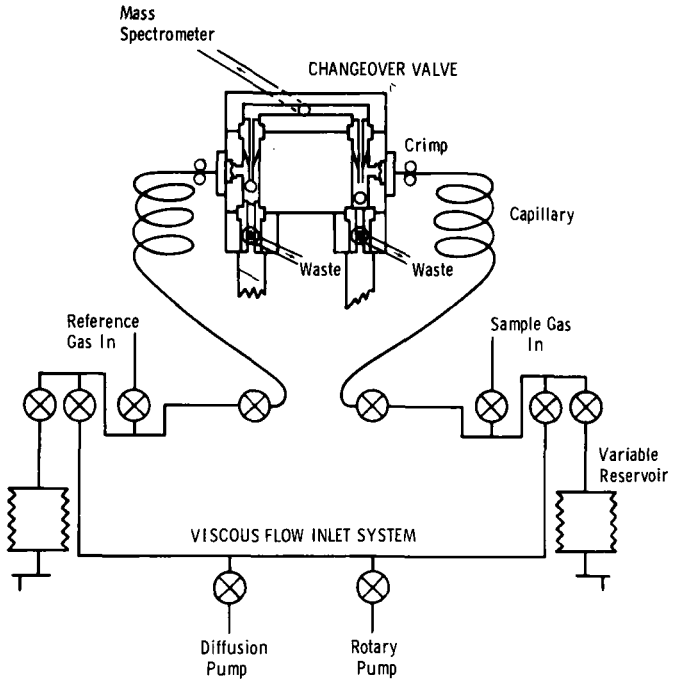


Figure 1.4. A typical viscous flow, dual gas inlet system required for precise isotope ratio measurement

though the characteristics change from viscous to molecular flow on passing the crimped region of the capillary, so that the gas entering the ion source has an isotopic composition identical to that in the gas reservoir [77].

The ion source. This constitutes a gas-tight region permitting a pressure differential to be created between the source chamber and the ambient vacuum. Electrons emitted from the filament describe a helical orbit, under the influence of small source magnets, providing the maximum chance of collision with sample gas molecules. This in effect produces maximum efficiency of ionisation.

Figure 1.3 shows the general construction of a typical source and collector assembly housed within the analyser tube. The electron beam is normal to the ion trajectory, has an energy of 80–100 eV and a total current of some 300–500 μA. Electrons not involved in collisions are collected by a trap whose resultant current provides an indication of the efficiency of ionisation when compared to the total source current. The ion repeller, which is positive with respect to the source, directs the ion beam towards the exit slit. The remaining plates in the source assembly contain slits that progressively focus the ion beam leaving

the source. The applied voltages to these plates are variable and their setting is dependent on the isotopic species to be analysed. The final alpha plate defines the maximum beam width at that point prior to its passage along the flight tube to the collectors.

The ion collectors. High precision isotope mass spectromers have double collectors arranged either in plate form as in the original Nier type instrument [78] or as independent Faraday buckets. These configurations permit the separate, simultaneous collection of the two isotopic ionic species whose ratio is required. In the Nier system, the major ion beam is collected on the first plate which contains a slit of sufficient width to permit the minor ion beam to pass through and impinge on the second collector plate. The Faraday bucket collector system has major and minor resolving slits spaced before the buckets. Electron suppressor electrodes (−50 V) are positioned between these components to minimise ion drift between the two collectors and to reject secondary electrons formed by ion bombardment.

The currents carried by the major and minor ion beams are of the order of 10^{-9} and 10^{-11} A respectively. These pass to the major and minor head amplifiers that have high impedence, low current noise characteristics and are normally of d.c. vibrating reed design. High value feedback resistors in parallel with the head amplifiers ensure maximum gain. Treatment of the output voltage from the head amplifiers is considered in the following section.

Measurement of the isotopic ratio. This is normally achieved using either a bridge–amplifier balance system or the infinite bridge null system. The former method provides that the output of the major amplifier supplies a resistor divider network whose output is fed back to the input of the minor amplifier.

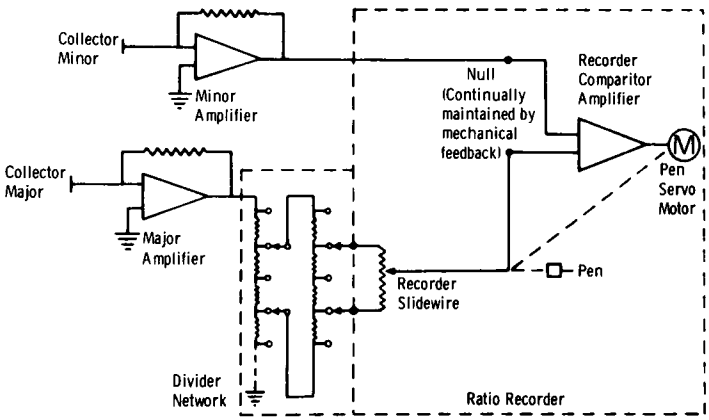


Figure 1.5. The infinite bridge null system for isotope ratio measurement

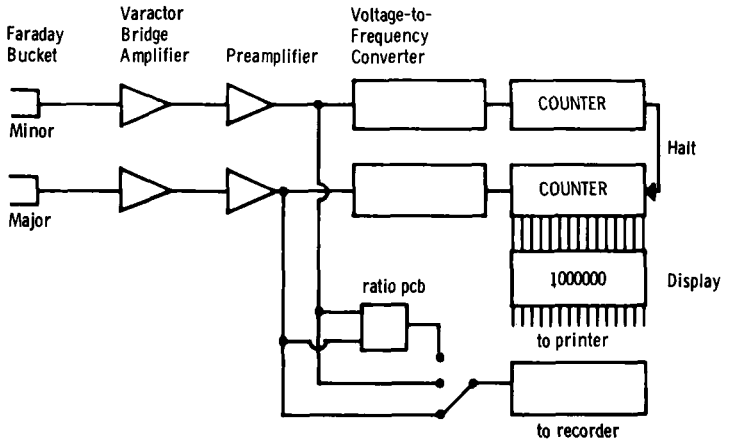


Figure 1.6. Direct counting bridge system for isotope ratio measurement

Adjustment of the divider to attain a given pen position on a potentiometric recorder provides the ratio from the sum of the divider and recorder readings.

The infinite bridge null system (*Figure 1.5*) is arranged such that the recorder slidewire forms part of the bridge measuring circuit and is thus an integral part of the resistor divider network. The output from both the divider network and the minor amplifier are fed to the recorder comparator and as the null point of the bridge is within the servo loop of the recorder, balance is obtained over the entire range of the recorder chart. In this instance chart calibration is directly related to the divider network and is independent of the signal levels.

A distinct advance in the direct measurement of isotopic ratios has recently been introduced to a commercially available isotopic mass spectrometer *. This new system is shown in *Figure 1.6*. In this instance ion beams are collected in Faraday buckets which are connected to major and minor beam varactor bridge amplifiers. Additional signal amplification is through preamplifiers whose signals are fed through voltage to frequency convertors. The generated pulses, whose repetition rates are linearly proportional to the input voltages, are fed to separate major and minor counters. These counters are set to zero and started simultaneously. When the major counter reaches 10^6 the minor counter is automatically stopped. This system provides directly the ratio, minor ion beam : major ion beam. The distinct advantage of this measuring system is that the ratio obtained in independent os sample size, pressure or depletion rate.

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* V.G. Micromass 602, Nat Lane, Winsford, Cheshire CW7 3BX, England.

Expression of enrichment. As indicated previously an isotope mass spectrometer is used to compare the relative isotopic enrichment of a sample gas with a reference gas. Absolute isotopic content cannot be obtained from these measurements.

The reference gas of choice is dependent on the type of investigation undertaken. Environmentalists such as geochemists, petrologists or oceanographers are involved in the determination of naturally occurring variations of a specific isotopic species. In order that interlaboratory results should be strictly comparable it is essential that the reference gas used should be identical in all laboratories. To this end international standards of known isotopic content are available from the International Atomic Energy Agency in Vienna.

These investigators have always expressed their results in the $\delta\text{‰}$ notation as the deviation per mil (parts per thousand) of the isotopic ratio of the sample compared to that of the reference gas. Thus in terms of ^{13}C measurements:

$$\delta^{13}C = \frac{^{13}C/^{12}C \text{ sample} - {}^{13}C/^{12}C \text{ standard}}{^{13}C/^{12}C \text{ standard}} \times 1000$$

In biological and clinical investigations the position is somewhat different. In both instances the enrichments measured are often large and it is normal practice to express the enrichment of a particular fraction as compared to the same fraction prior to the introduction of the labelled compound to the system. In such cases most workers express enrichment of isotopic content as either atom% or atom% excess which have been defined earlier (p. 5). Where ^{15}N is used at low enrichments and measured in an isotopic ratio instrument normally equipped to monitor the *m/e* 28 and *m/e* 29 ion beams, the following formula is applicable:

$$\text{atom\% } ^{15}N = \frac{100}{2[I_{28}/I_{29}] + 1} \quad \text{where } I_x = \text{intensity of } m/e\ x.$$

At higher enrichments modification of this formula is required to account for the increasing contribution of the *m/e* 30 ion species.

Increasing interdisciplinary collaboration in the area of isotopic analyses, stemming from common instrumentation and sample preparation schemes, suggests that standardisation of terminology would be advantageous. For the sake of clarity and uniformity one might hope that the rather cumbersome 'atom% excess' nomenclature cease to be used.

Analytical accuracy (sample size and handling). It is impossible to be dogmatic concerning the absolute accuracy of isotopic measurement obtainable

Table 1.1. CONDITIONS AND EXPECTED ACCURACY FOR THE MEASUREMENT OF THE ISOTOPIC CONTENT OF A PURE GASEOUS SAMPLE

Isotope	*Gas analysed*	*Sample size required (atm cm^3)*	*Accuracy of measurement (δ ‰)*	*Gas transfer into mass spectrometer*
D	H_2/HD	0.2	±0.5–1.0	uranium 80°, charcoal
^{13}C	CO_2	0.01–0.05	±0.1	liq. N_2
^{15}N	N_2	0.05–0.10	±0.1–0.2	liq. He, molecular sieve
^{18}O	CO_2	0.01–0.05	±0.1	liq. N_2
^{34}S	SO_2, SF_6	0.05–0.10	±0.2	liq. N_2

from modern spectrometers. This results from the fact that sample preparation, gas handling and instrumental errors for a composite system and the final accuracy obtained will be a reflection of the additive component errors. With experience, it is possible to obtain accuracies for overall isotopic analyses shown in *Table 1.1.* For convenience and interest, the table also includes typical sample size requirements and methods of sample introduction into the mass spectrometer. An automatic Toepler pump or bellows system can be used to compress all gases into the sample reservoir.

Gas chromatography–mass spectrometry (selected ion monitoring)

Detailed coverage of gas chromatography–mass spectrometric analysis, as applied to clinical investigation, has been largely excluded from this review, though pharmacological studies employing stable isotopes are committed to organic mass spectrometry for analytical purposes. However, attention should be drawn to a specific mode of analysis that is relevant to both quantitative and structural studies involving isotopically labelled components. Selected ion monitoring (SIM) provides for the focusing of ions of selected masses at the detector of a magnetic deflection instrument by switching the accelerating voltage through pre-set increments, in a cyclical mode. The limitations of the method are the time required for switching between selected masses and the range through which the accelerating voltage can be altered without the ion source becoming defocused. The high degree of stability required for the ion focusing systems is normally achieved using computer based feedback [79]. Computer systems are increasingly used to record and process data obtained from SIM analyses [80,81]. The advantages of this analytical system are the requirement of only 0.1–10 ng of material and the detailed information provided concerning the quantitation and structural position of the isotopic label.

In clinical investigations, the use of SIM for isotope dilution studies provide an accurate reference method for a specific assay against which routine laboratory analytical procedures may be standardised [82]. A comprehensive review of SIM has appeared recently [83].

Sample preparation for isotopic analysis

Part of any clinical investigation involving the use of stable isotopes is the preparation of a final gaseous sample from a variety of starting materials that result from the investigation undertaken. This sample preparation should be conducted in a manner such that isotopic fractionation is precluded at each stage. This is most likely to occur in the preparation of H_2/HD gas as these two isotopes exhibit the largest mass difference within a given species.

It seems appropriate therefore to consider briefly the preparation of hydrogen, carbon dioxide and nitrogen required for the isotopic analysis of ^{3}H, ^{13}C and ^{15}N content respectively. The preparation of carbon dioxide and sulphur dioxide for analysis of ^{18}O and ^{34}S content will not be considered in detail as these isotopes have as yet very limited application in a clinical context.

Deuterium. Analysis of the deuterium content of a biological fluid or the water of combustion derived from organic material is performed on hydrogen gas resulting from the quantitative reduction of the aqueous sample. This reduction is normally performed in a separate preparation line [84,79] using zinc [85], magnesium [86], or uranium [84], at 400°C, 600°C and 800°C respectively, as the reductant. The H_2/HD gas is then pumped into an appropriate sample bottle by means of an automatic Toepler pump. Alternatively the H_2/HD gas can be reacted with finely cut uranium turnings at 80°C to form the hydride (UH_3). On completion of the reduction, the hydride is decomposed by heating to 800°C, liberating the hydrogen gas into the sample bottle [87]. This latter method of hydrogen transfer is simpler and safer than the use of a mercury Toepler pump. Modifications to this preparation scheme have been developed for 'on line' conversion of microgram quantities of water to hydrogen gas which is admitted directly into the mass spectrometer source region [88]. Recently it has proved possible to use a tapered capillary probe as the initial water sample inlet system whereby a rapid liquid to water vapour conversion occurs without isotopic fractionation [89]. A further modification using a hot probe inlet system has been described that exploits the 'Leidenfrost' effect and permits the analysis of impure water samples [90]. In both instances reduction of the water vapour is performed between the capillary leak and the spectrometer source by heated uranium ribbons in the line.

The precise estimation of deuterium afforded by modern isotope mass spectrometers suggests the possibility of employing deuterated precursors in a

range of quantitative clinical metabolic studies. Following initial separation and purification, specific compounds may be combusted along classical lines to yield water and carbon dioxide [86,91]. The former may then be converted to hydrogen gas by one of the methods outlined above.

Carbon-13. The increasing availability of ^{13}C-labelled substrates has provided a marked impetus in the use of these tracers in clinical studies. Most recent interest centres round the use of $^{13}CO_2$ breath tests for the *in vivo* estimation of the rate of oxidation of specific ^{13}C-labelled substrates. Some clinical implications of these tests will be discussed later. Mention should be made of the available methods for respiratory CO_2 collection. They include the collection of whole breath in evacuated glass tubes prior to CO_2 separation [92], the direct cryogenic trapping of exhaled CO_2 in liquid nitrogen [93] and the precipitation of CO_2 as carbonate in sodium hydroxide [94]. The latter method is suspect in that it results in isotopic fractionation which may not be reproducible.

The availability of ^{13}C-labelled glucose has provided the means of investigating glucose flux and turnover in human subjects. When the ^{13}C overall isotopic content of glucose is required, conversion to $^{13}CO_2$ can be achieved by a wet combustion *in vacuo* of gluconic acid derived from the glucose by enzymatic oxidation. An alternative procedure, if [1-^{13}C]glucose is the substrate involved, is that of enzymatic decarboxylation [95]. Utilizing this system it is possible to demonstrate experimentally that either the $^{13}C/^{12}C$ of the evolved CO_2 at equilibrium is the same as that of the carbon-13 of the initial glucose sample or that if isotopic fractionation occurs, it is of constant magnitude providing the carbon recovery exceeds 60%.

The estimation of the ^{13}C content in carbonate, resulting from $^{13}CO_2$ trapping, involves the reaction with 100% phosphoric acid under rigorously controlled conditions originally described over 25 years ago [96].

Finally, organic samples requiring analysis of ^{13}C isotopic content, may be combusted in a stream of oxygen to provide the carbon dioxide for isotopic analysis. Several combustion systems have been used that differ only in minor components designed to remove contaminants such as sulphur or the halogens [97,98].

In ^{13}C isotopic analysis where the greatest accuracy is required, correction must be made for the contribution of $^{12}C^{16}O^{17}O$ to the mass 45 peak in reference and sample gases [99].

Nitrogen-15. Despite the increasing use of the heavy isotope of nitrogen not only in clinical investigations but also in animal and agricultural studies, analytical techniques have not developed to any marked degree over the last few decades. It is obligatory that gaseous nitrogen is prepared for ^{15}N analysis in an isotope mass spectrometer. Two general methods are available for the conversion of an array of N-containing compounds to N_2 gas. These are the Dumas

direct combustion technique, (which with modification, forms the basis of many commercial nitrogen analysers) and the Kjeldahl–Rittenberg technique [100]. The initial step in the latter technique is an acid digestion to convert all nitrogenous components to the ammonium state. The use of a variety of catalysts and salt additives to elevate the boiling point of the digestion mixture have been discussed with specific relevance to biological materials [101]. The ammonium salt resulting from this digestion is recovered and oxidised to molecular nitrogen by the action of sodium or lithium hypobromite. This final oxidation reaction, originally performed in Rittenberg tubes, has been recently modified so that the final oxidation may be semi-automated [102–103]. Critical appraisal of both methods for the production of nitrogen gas including inherent difficulties has been presented [104], as have corrections required for atmospheric contamination of samples [105].

Oxygen-18. The most widely employed method for determination of oxygen isotopic content of water is equilibration with CO_2 followed by analysis of the CO_2 isotopic ratio [106,107]. For the most accurate ^{18}O measurements, corrections must be made for the differing ^{13}C and ^{17}O content of the reference and sample gas [99] and also to account for dissolved cations where relevant [108]. Alternative methods have been developed for measuring the oxygen isotopic content of water, where only microlitre samples are available [109]. Though technically more demanding, these two methods measure the $^{18}O/^{16}O$ ratio of the original water sample without recourse to a fractionation factor required in the CO_2 equilibration method. The use of guanidine hydrochloride originally detailed for the conversion of oxygen of orthophosphate or water to CO_2 for ^{18}O analysis, has promise of wider applications [110]. To measure the ^{18}O content in organic samples requires initial combustion by an appropriate method to provide CO_2 for isotopic analysis.

Sulphur-34. The analysis of ^{34}S content in a prepared sample normally requires initial conversion to sulphur dioxide, though sulphur hexafluoride resulting from the fluorination of sulphide has been used for mass spectrometric analysis [111,112].

As with other procedures for the production of a gas sample for isotopic analysis, caution is required to ensure complete conversion at each stage of the preparation to obviate kinetic fractionation. Sulphides may be oxidized directly to SO_2 for isotopic analysis [113] and recently methods have been developed for the thermal decomposition of sulphates [114,115]. An alternative approach is the initial conversion of either sulphate or sulphide to hydrogen sulphide [116, 117] from which the sulphur is precipitated as silver sulphide. This latter sulphide is easily oxidised in a reproducible manner to provide pure SO_2 for isotopic analysis.

NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

Several of the isotopes with which this review is concerned have a non-zero spin and can therefore be detected by nuclear magnetic resonance (NMR) spectroscopy. Carbon-13 and nitrogen-15 have nuclear spins of 1/2 and oxygen-17 of 5/2. Of these heavy isotopes, the overwhelming mass of the work reported has concerned carbon-13.

It is not proposed to discuss the technique of NMR spectroscopy and its use in combination with stable isotopes in detail as many reviews and monographs are available, several of which specifically deal with its application to biological and biosynthetic problems [118–125]. Using ^{13}C-NMR, carbon-13 can now be detected at the sub-microgram level.

In early applications, information on the position of enriched carbon-13 atoms in a molecule employed proton NMR and the so-called satellite method. Hydrogen atoms attached to a carbon-13 atom show spin coupling and therefore each proton resonance in the NMR spectrum is accompanied by 2 satellite bands. In a spectrum of a molecule with natural abundance carbon-13, the intensity of these signals is very low and they are not normally observed in a single sweep. However, with enriched carbon-13 at certain sites in the molecule, the satellite signals corresponding to the labelled sites will be enhanced providing the carbon-13 has hydrogens attached. The method, although useful, has severe limitations, In many cases, the main proton bands may overlap the satellite signals. Nevertheless, the difficulties can often be overcome and many successful studies of biosynthetic pathways have been carried out using this indirect approach and these have been reviewed [122,126].

However, the direct measurement of carbon-13 is clearly preferable. The basic problem was that, compared with proton NMR, the sensitivity of ^{13}C-NMR was lower by a factor of about 6000, but this was overcome with the introduction of Fourier transform NMR [127]. Conventional frequency NMR is extremely inefficient since only one frequency is observed at any given instant. However, using a short pulse radiofrequency, all the ^{13}C nuclei in a sample can be excited simultaneously. The absorption of individual frequency components by each nucleus are detected by the receiver and these are abstracted by Fourier transformation using data acquisition and processing equipment. The use of ^{13}C-NMR as a tracer, especially in biosynthetic studies, has now become a very versatile tool. Not only can the ultimate fate of carbon-13 be determined, but unlike its radioactive counterpart, carbon-14, the location of the label and the molecular structure can often be assigned without the necessity for degradation of a complex compound. As a cross check, the level of carbon-13 incorporation may frequently be confirmed by mass spectrometric analysis.

Other isotopic tracers may also be investigated using ^{13}C-NMR. For exam-

ple, the substitution of a hydrogen atom with deuterium produces characteristic perturbations in the ^{13}C spectra so that the carbon atom bearing the deuterium may be readily identified. Work of this type has been reviewed [123].

In NMR studies, the substance must be dissolved in a suitable solvent which in most cases will need to be deuterated. A whole range of such solvents is available and where the natural abundance ^{13}C resonances in the solvent obscure vital signals from the compound being examined, deuterated solvents depleted in carbon-13 (^{12}C-deuterated solvents) can be employed. The carbon-13 content of these solvents has been depleted to 0.05–0.1 atom% ^{13}C and somewhat surprisingly, there is no observable signal when they are examined by Fourier transform ^{13}C-NMR [128,129].

Although the possibility of using carbon-12 as a negative label has frequently been suggested, the incorporation of ^{12}C precursors in a biological process being detected by a reduction in the ^{13}C natural abundance signal [130], the impracticability of such a technique in biosynthetic and metabolic studies has been pointed out [125]. Justification for such use on grounds of cost is somewhat nebulous, since on a relatively small-scale, the bulk of the cost of the labelled precursors resides not so much in the price of the isotope as in the labour intensive chemical synthesis. However, this is not to say that negative labelling does not have a role to play. In simple problems of a mechanistic nature involving the use of an easily synthesized labelled compound, it could offer advantages.

^{15}N-NMR has not as yet been used to the extent of ^{13}C-NMR in labelling studies with stable isotopes. Work on both ^{14}N-NMR and ^{15}N-NMR has been reviewed [131,132]. Nitrogen-14 nuclei possess a nuclear quadrupole moment that significantly broadens their NMR resonances. Although nitrogen-15 spectra display excellent resolution, poor signal to noise ratios have restricted the use of ^{15}N-NMR studies. Fourier transform ^{15}N-NMR spectra have been published for an algal amino acid mixture, a chlorophyll pigment mixture, glycylglycine, and haemoglobin, all labelled with nitrogen-15 [133]. The use of ^{15}N labelling and the application of ^{15}N-NMR has been reported in extensive studies in molecular haematology [132]. The development of ^{15}N-labelling techniques in this work has made it possible to observe the ^{15}N resonance of macromolecules. It has therefore been suggested, that due to the potentially greater range of ^{15}N chemical shifts and the narrower line widths of polypeptide ^{15}N nuclei, ^{15}N-NMR should have particular interest in the investigation of protein structure [134].

Both ^{13}C-NMR and ^{15}N-NMR have been applied to the study of living cells. The anaerobic metabolism of [1-^{13}C]glucose to [2-^{13}C]ethanol by yeast has been studied by ^{13}C-NMR spectroscopy of living cells [135]; living cells of the fungus *Ustilage sphaerogena* grown in [^{15}N]ammonium acetate were studied by ^{15}N-NMR [136].

As yet, ^{17}O-NMR has not assumed an important role in biochemical studies

but this is an area that will no doubt prove of interest and importance in the future.

Some specific applications of NMR spectroscopy to biological problems will be referred to in subsequent sections.

OPTICAL EMISSION SPECTROSCOPY

Optical emission spectroscopy is routinely used for the determination of nitrogen-15. The method depends upon the wavelength separation of the isotopic species of nitrogen gas ($^{14}N_2$, $^{14}N^{15}N$, and $^{15}N_2$) due to the isotopic shift. Prior to assay, it is necessary to convert the nitrogen to nitrogen gas. For example, amino compounds are treated with hypobromite. The Statron NOI 5 analyser based on this principle has been in use for a number of years. Although, compared to the isotope ratio mass spectrometer, more tracer is required for results of comparable accuracy, it has been used to determine urinary [^{15}N]urea, [^{15}N]ammonia, and ^{15}N-labelled amino acid on infusion of [^{15}N]glycine into human subjects [137]. More recently, sample conversion, optical detection, and electronic calculation have been combined in one instrument, the Isonitromat 5200. With a sample requirement of 120 μg of nitrogen, its use has been demonstrated in clinical metabolic studies using [^{15}N]glycine as a tracer [138]. The relative accuracy was ±1% over an ^{15}N range of 0.36–25 atom%.

INFRARED SPECTROSCOPY

Infrared spectroscopy has limited applications in the detection and assay of labelled compounds. Absorption peaks at 2193 cm^{-1} for carbon monoxide-^{12}C and at 2144 cm^{-1} for ^{13}CO are sufficiently separated to determine the isotope ratio [139]. With ^{13}C contents ranging from 1 to 20 atom%, the mean error quoted was about 2% and had a relative standard deviation of about 6%. [*Carbonyl*-^{12}C]acetophenone gives a peak in the infrared spectrum at 1685 cm^{-1} while the (*carbonyl*-^{13}C)-labelled compound has a corresponding absorption at 1645 cm^{-1} [128]. In certain cases it is therefore possible to utilise published data for a rapid assay of labelled compounds. Infrared data on ^{13}C- and ^{15}N-labelled compounds have been reviewed [140].

MISCELLANEOUS METHODS

Although as yet not widely used, the possibility of using Raman scattering for measuring isotope ratios has been investigated. It has been demonstrated that

isotopic analyses of nitrogen ($^{14}N^{15}N$) and oxygen ($^{16}O^{18}O$) can be made to accuracies of at least ±0.1% using an argon laser [141].

Activation analysis is another technique that has found some applications. In these methods, the desired stable isotope is made to undergo a suitable nuclear reaction. The parent isotope is determined by measurement of the resulting nuclide. As an example, the ^{18}O content of water samples as small as 1.5 μl has been determined by charged particle activation [142].

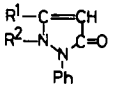
PHARMACOLOGY

It has been pointed out [143] that one of the most pressing problems of pharmacology is the acquisition of data on the variation in drug disposition between patients. Not only is drug efficacy a matter for concern but also the problems that occur as a result of adverse reactions to drug administration. The use of stable isotopes in pharmacological research is now well-established as an aid to obtaining quantitative and structural information on the metabolism of drugs and derives from the fact that mass spectrometry coupled with gas chromatography is a powerful tool in such studies. Identification and structure analysis of metabolites, as well as their qualitative and quantitative detection, can be achieved at sub-nanogram levels in biological materials. The versatility of the techniques is enhanced by the availability and use of stable isotopes.

Stable isotope labels are used in tracer studies, to follow the pathway of a drug, or else, but equally important, to distinguish between a true drug metabolite and a naturally occurring substance in a biological sample. But probably the most widespread use to date has been as an internal standard for quantitative gas chromatography–mass spectrometry. Addition of a stable isotope labelled drug or metabolite in known amount to a mixture enables a quantitative assessment of the natural compound to be made from the mass spectral data.

The use of stable isotopes in pharmacology and drug research has been reviewed [8,68,144–147]. Most of the work reported has utilised deuterium labelling and undoubtedly this has largely been dictated by economic factors. However, there are occasions when its use is limited. In certain chemical situations, the deuterium atom is readily exchanged; for efficient use in quantitative work, and often in qualitative work, there must be no possibility of isotopic exchange in the environment in which the labelled compound is used. This limits the use of oxygen-18 labelling but does not pose a significant problem with carbon-13 and nitrogen-15.

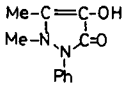
Metabolic switching has been reported as a consequence of deuterium labelling where a drug is metabolised by multiple alternate pathways rather than sequentially [20,21]. Both *in vivo* studies in rats and *in vitro* studies using rat



(6a): $R^1 = R^2 = Me$

(6b): $R^1 = CD_3$, $R^2 = Me$

(6c): $R^1 = Me$, $R^2 = CD_3$



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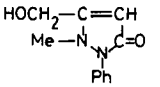

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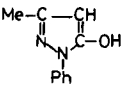


(9)

liver homogenates were used in a comparison of phenazone (antipyrine) (6a) and two methyl deuterated analogues (6b and 6c). 4-Hydroxyantipyrine (7) and the 3-hydroxymethylantipyrine (8) were the major metabolites of antipyrine (6a) and their N-CD_3 analogues from the N-CD_3 compound (6c). However, the metabolism was switched from hydroxylation of the C-3 methyl group to N-demethylation (formation of compound 9) by deuterium substitution in the 3-methyl group. Metabolic switching as a consequence of deuterium substitution was also demonstrated in caffeine [20] and pethidine (meperidine) [21].

As the cost differential narrows, especially of synthesized compounds, and taking the other factors mentioned above into consideration, it seems likely that carbon-13 and/or nitrogen-15 labelling will become the preferable choice where practicable in future metabolic studies.

Many of the disadvantages of stable isotope labelling as compared with radiolabelling stem from limits on the sensitivity which arise from the natural abundance background. Using multiple labelled compounds as tracers, this problem can be overcome and furthermore, an assessment may be made of isotope effects in the system under study. The use of multiple labelled compounds and methods of detection allowing the determination of 1 pg in a 1 μg sample have been reviewed [65].

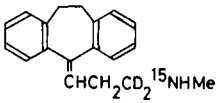
IDENTIFICATION OF METABOLITES – STABLE ISOTOPES AS TRACERS

By incorporation of a known percentage of a stable isotope labelled drug (normally deuterium, carbon-13, nitrogen-15, or oxygen-18) the relevant metabolites can readily be detected in a biological isolate, without the need for extensive

purification and isolation procedures from the isotope clusters that are produced in the mass spectrum [148]. The technique of mass fragmentography uses the mass spectrometer as a highly sensitive specific detector for a limited number of ions. The detection of specific labelled ions can be made quantitatively at the sub-nanogram level. To gain the maximum advantage, the labelling should be in a position in the molecule where it is largely resistant to biotransformation and as many metabolites as possible will be labelled.

This technique for the location and structural identification of metabolites was demonstrated by studies on nortriptyline and trideuterium labelled nortriptyline; the drug, and any metabolites retaining the labelled site, showed a doublet M, M + 3 for the molecular ion and any fragment retaining the label [149]. Although first demonstrated with deuterium, other stable isotopes have been used in this type of work. In deciding on a labelling pattern to be adopted, it must be remembered that the utility of a M, M + 1 doublet is limited by the effects of the contribution of the natural abundance isotopic species.

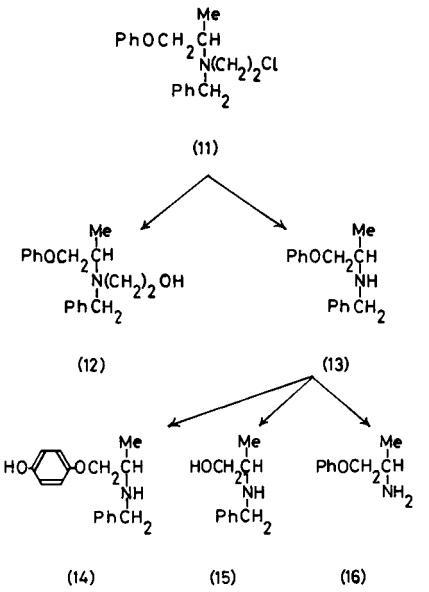
The importance of studying metabolites that occur in very low concentrations in urine has been underlined since the more reactive a species, the lower its concentration may be [143]. Mixtures containing dideuterium-nitrogen-15-labelled nortriptyline (10) have been used to study urinary and biliary metabol-



(10)

ites. The use of mass fragmentography facilitated the study of metabolites that were present in too small a quantity to be detected by scanning of the mass spectra [150]. Subjects received a single 25 mg dose of equimolar amounts of labelled and unlabelled drug as their hydrochlorides. Metabolites were identified from the M, M + 3 doublets when trifluoroacetylated extracts were examined by gas chromatography–mass spectrometry. Unchanged drug, 10-hydroxynortriptyline, desmethylnortriptyline, and 10-hydroxydesmethylnortriptyline were identified in human urine and bile after a single oral dose of nortriptyline, thus demonstrating the sensitivity and safety of the technique [150]. The usefulness of these clusters to select correct empirical formulae from exact mass measurements has been demonstrated [145]. In favourable circumstances, pg levels can be identified.

In more recent work along the lines discussed above, new nitrogen containing metabolites of phenoxybenzamine (11) were identified by administration of an equimolar mixture of the drug and the ^{15}N-labelled drug to rats and dogs. Uri-



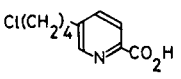
Scheme 1.3. Nitrogen-containing metabolites of phenoxybenzamine

nary metabolites were examined by gas chromatography–chemical ionisation mass spectrometry. The only previously identified metabolite was the alcohol (12); however, the present work identified 4 further metabolites, (13, 14, 15) in rats and (13, 14, 16) in dogs [151] (*Scheme 1.3*).

Metabolic studies have recently been reported on the antibacterial agent cinoxacin, 1-ethyl-1,4-dihydro-4-oxo[1,3]dioxolo[4,5-*g*]cinnoline-3-carboxylic acid. The compound was synthesized with both nitrogens labelled with nitrogen-15 at greater than 99 atom%, and, in an equimolar mixture with unlabelled drug, was administered to humans. The doublet arising from the isotope labelling in gas chromatography–chemical ionisation mass spectrometry results proved an excellent aid in the identification of urinary metabolites [152].

Labelling with carbon-13 followed by mass spectrometric analysis of the metabolic products has also been successfully used to identify metabolites [68, 153]. 5-(4′-Chloro-*n*-butyl)picolinic acid (CBPA) (17), which inhibits dopamine β-hydroxylase and also shows a hypotensive effect, showed an unexpected metabolic pathway when administered to rats. A 2-carbon chain elongation occurred in the 2-carboxyl group of the pyridine ring. Using carbon-13 labelled CBPA, the existence of the same metabolic pathway was demonstrated in man showing the utility of single labelling, notwithstanding the contribution from the

natural isotopic abundance [153]. However, to attain the maximum advantage from the use of M, M + 1 combinations using carbon-13 labelling, techniques have been developed to increase sensitivity and overcome the problems of natural abundance peaks. The presence of a carbon-13 labelled tracer could be determined in urine after extraction of the metabolites by measuring the excess carbon-13 by isotope ratio mass spectrometry after combustion to carbon dioxide. In trial experiments with aspirin (*carboxyl*-^{13}C) metabolites were detected in a 24 h urine sample after a 320 μg dose of labelled drug. It was concluded that 5×10^{-5} g excess ^{13}C per g carbon should be provided in any fraction to be analysed [154].



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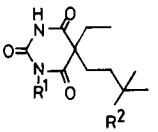

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A subsequent technique involved pyrolysis of the metabolites after gas chromatography. The $^{13}CO_2 : {}^{12}CO_2$ ratio was determined by mass fragmentography. Metabolites in a 24 h urine fraction were detected from administration of doses of ^{13}C-aspirin of less than 100 ng [155,156].

The simultaneous labelling of a drug with a carbon-14 radioactive label and a carbon-13 stable label at the same position has also been employed. The metabolism of V-K 774 labelled at C-2 (3) was studied in the rat. The quantitative determination of the metabolites in the urine was made from the radioactive studies and structural identification was achieved from the mass spectra using the isotopic clusters to differentiate the metabolites from other biological impurities [69]. A combination of radio and stable isotope labelling has also been used to study the metabolism of amylobarbitone (amobarbital) (18,$R^1 = R^2 =$ H) in humans. Oral administration of [2-^{14}C]amylobarbitone showed that 80–90% of the radioactivity was excreted in the urine and 4% in the faeces. Administration of a 50% mixture of amylobarbitone and the $^{15}N_2$-labelled drug showed that 40% of the urinary excretion was as 3′-hydroxyamylbarbitone (18,R^1 = H, R^2 = OH). The majority of the remainder was the previously unrecognised *N*-hydroxyamylobarbitone (18,R^1 = OH, R^2 = H) and a minor component that was not identified. This was the first example of the N-hydroxylation of barbiturates [157].

STABLE ISOTOPE LABELLED COMPOUNDS AS INTERNAL STANDARDS

The purpose of an internal standard in analytical techniques is to correct for losses of a compound under study. There must therefore be both chemical similarity and similarity of behaviour between the standard and the compound subject to examination. When gas chromatography–mass spectrometry is employed in drug metabolism studies, mass differences alone can be used to discriminate between the compound analysed and the standard. Thus an isotopically labelled compound represents the ideal standard. This forms the basis of a most important application of stable isotopes in drug research.

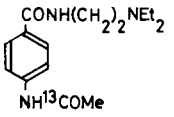
The use of deuterated nortriptyline and deuterated-[^{15}N]nortriptyline was investigated for the estimation of nortriptyline in plasma [158]. The labelled standard was added to the crude sample before gas chromatography–mass spectrometry. In other drug applications, the labelled drug has been added prior to extraction, derivatisation, and analysis [145].

Drugs in urine, plasma, breast milk, and amniotic fluid, analysed quantitatively in the picogram range, provide an opportunity to examine drug profiles of biological fluids. This has been achieved by selective ion detection using gas chromatograph–mass spectrometer–computer systems operated in the chemical ionisation mode, combined with stable isotope labelled drugs as internal standards [159]. In 50–200 μl samples of plasma and breast milk, [2,4,5-^{13}C]-diphenylhydantoin has been used to quantify phenytoin (diphenylhydantoin) and [2,4(6),5-^{13}C]pentobarbitone and [2,4(6),5-^{13}C]phenobarbitone to determine amylobarbitone (amobarbital), quinalbarbitone (secobarbital), caffeine, pethidine (meperidine) and phenobarbitone (phenobarbital) [159]. Even smaller plasma samples can be used to determine drugs in relatively high concentration (μgml^{-1}) [160]. The clinical applications of these analytical systems have been further reviewed [161] and factors affecting the choice of label, its position and the synthesis of the labelled drugs have been described [162]. Similar systems have been used in pharmacokinetic studies. In this work, which has involved plasma, urine, breast milk, saliva, and liver homogenates, stable isotope labelled drugs were added to biological samples before isolation procedures were carried out. Carbon-13 labelled standards included [2,4,5-^{13}C]phenobarbitone [2,4,5-^{13}C]diphenylhydantoin, and [2,4,5-^{13}C]pentobarbitone [163].

Analytical procedures have been developed utilising chemical ionisation mass spectrometry in conjunction with stable isotope labelled amines to obtain quantitative information on the metabolism of (*R*-), (*S*-), and (*R*,*S*-) methyldopa (alpha-methyldopa) and the regional concentrations of dopamine, α-methyldopamine, noradrenaline (norepinephrine) and α-methylnoradrenaline in rat brain. ^{13}C-labelled (*S*)-α-methyldopa, as well as deuterated amines, were employed. Isotope dilution techniques provided good quantitative data [164].

STUDIES OF BIOAVAILABILITY

To determine the absolute bioavailability of a drug normally requires two experiments in each subject involving the separate administration of an intravenous and an oral test dose. It must then be assumed that the drug kinetics remain unchanged between doses. By administering the drug orally, and simultaneously an intravenous injection of a stable isotope labelled version of the drug, these disadvantages can be overcome. Furthermore, absolute bioavailability in each subject can be determined from a single study and analysis of only one set of blood samples [165,166]. The feasibility of the approach has been demonstrated on studies of *N*-acetylprocainamide (NAPA). [^{13}C]NAPA (19) was



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injected intravenously at the same time as the unlabelled drug was given orally. Plasma and urine levels were determined by mass fragmentography using [D_5]-NAPA as an internal standard [165,166].

STABLE ISOTOPES IN COMPOUNDS OF THERAPEUTIC INTEREST

A recent novel use of stable isotopes in an area of interest to the medicinal chemist has been to increase the therapeutic index of an antibacterial agent.

$CH_2F.CD(NH_2)CO_2H$
(20)

3-Fluoro-D-[2-^{2}H]alanine (20) shows the same antibacterial activity as the protio form of the fluoroamino acid. However, since the metabolism *in vivo* is several fold less substitution of the deuterium produces enhanced pharmacological activity [167].

This is an area that is really in its infancy, but it may well produce significant advances in the future.

STABLE ISOTOPES IN BIOCHEMICAL STUDIES

To define where the borderline between biochemistry and medicinal chemistry occurs is an invidious task. So far as the medicinal chemist is concerned, the

main interest is in the metabolism of drugs, i.e. compounds that are alien to the animal system. This aspect has been discussed in the previous section. Metabolic studies may also involve natural compounds and within the limitations of space it is proposed to briefly discuss the role of stable isotopes in such biochemical investigations.

Extensive studies involving the use of stable isotopes had taken place on the metabolism of proteins and the intermediary metabolism of carbohydrates and lipids by the mid-1940s and were reviewed in 1947 [1]. Much of the work reported was not only of immense biochemical interest but also provides useful data on the synthesis of labelled compounds such as amino acids [73]. General considerations on the study of protein metabolism were reported [168] and it was shown that nitrogen in amino acids does not exchange with other compounds in aqueous solution [169]. Specific metabolic studies in the rat used for example, [^{15}N]aspartic acid [170], L-[^{15}N,D]proline [171], and L-[^{15}N,D]-leucine [172], and nitrogen-15 labelling was used to study the metabolism of lysine in the rat [173]. The design of experiments for the study of nitrogen metabolism in ruminants with the aid of nitrogen-15 labelled compounds, and the interpretation of the results has been discussed [174].

Carbon-13 labelling has been utilised in studies of valine metabolism. [3-^{13}C]-Valine was administered to fasted rats and the glycogen formed was isolated; degradation confirmed the formation of a 3-carbon intermediate [175]. A patient with methylmalonic acidemia has been studied by administration of [2-^{13}C]valine and [2,3-^{13}C]valine; methylmalonic acid was isolated from the urine and analysed by ^{13}C-NMR [176,177].

Enzyme synthesis has been demonstrated using density labelling of proteins with deuterium, with nitrogen-15, and using triple labelling with deuterium, carbon-13, and nitrogen-15 followed by equilibrium density gradient sedimentation [178]. Heavy labelling of bacteriophage with nitrogen-15 and carbon-13 followed by density gradient centrifugation was used to determine the distribution of labelled DNA [179]. The replication of DNA in *Escherichia coli* was studied by density gradient centrifugation following the transfer of nitrogen-15 substituted growing *E. coli* to a natural nitrogen-medium. It was concluded that the nitrogen of a DNA molecule is divided equally between two physically continuous sub-units and following duplication, each daughter molecule receives one of these. Sub-units are conserved through many generations [180].

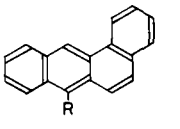
[^{13}C]Ethanol has been used to study ethanol metabolism in rats [181]. After administration of the [^{13}C]ethanol to bile fistula rats, the bile was collected and bile acids analysed by gas chromatography–mass spectrometry and by NMR [182,183].

The value of bile acids labelled with carbon-13 in gastroenterological research has been critically evaluated and compared with radioactive tagging. Stable iso-

tope labelling is essential in certain cases including studies to follow specific activity decay of the bile acid pool from serum samples where the concentration of bile acids in blood is so low and the pool itself is so large that the acceptable level of radioactivity administered would be inadequate [184]. As a generalisation, it can be stated that pharmacological studies involving large body pools and low plasma levels for measurement cannot be conducted without the use of compounds labelled with stable isotopes [185].

The enzyme *Candida utilis* 6-phosphogluconate dehydrogenase with carbon-13 enriched to 90 atom% has been obtained by growing the yeast with ^{13}C-labelled acetic acid as the sole carbon source [186]. Carbon-13 composition had very little, if any, effect on the catalytic properties of the enzyme. Sodium [^{13}C]lactate has been used to study the conversion of lactate to liver glycogen in the rat. Lactate labelled with ^{13}C at position 2 and ^{14}C at position 3 was also used. Carbon-13 was measured by mass spectrometry [187]. Similar experiments were carried out with ^{13}C-labelled propionate and ^{13}C- and ^{14}C-labelled propionate [188].

Mass spectra, ^{13}C-NMR, and ^{15}N-NMR have been applied to the study of isotopically labelled compounds of haematological interest. ^{13}C, ^{15}N-labelled haemoglobins, ^{15}N-labelled protoporphyrin-IX and coproporphyrin-III, and ^{13}C- and ^{15}N-labelled globins have been investigated [134].



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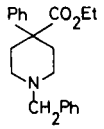

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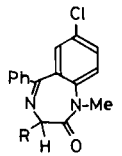

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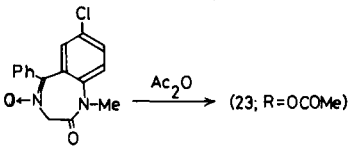

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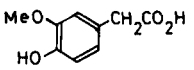
Incubation of cholesterol with a bovine adrenocortical mitochondrial acetone-dried powder preparation in an oxygen-18 enriched atmosphere provided evidence that hydroxyl groups in the side chain of (22*R*)-22-hydroxycholesterol, and (20*R*,22*R*)-20,22-dihydroxycholesterol, and that the C-20 oxygen atom of pregnenolone, originated from molecular oxygen [189].

Incubation of 7-methylbenz[a]anthracene (21, R = Me) with rat liver microsomes in ^{18}O-labelled water resulted in no incorporation of oxygen-18 into the hydroxymethyl metabolite (21, R = CH_2OH) whereas oxygen-18 incorporation did occur when it was incubated with oxygen-18 gas showing that the hydroxylation is a true oxygenase reaction [190]. The incorporation of oxygen-18 into benzaldehyde formed from the microsomal oxidative dealkylation of ethyl 1-benzyl-4-phenylpiperidine-4-carboxylate (22) in the presence of oxygen-18 gas, showed that the oxidative metabolism of nitrogenous bases follows a pathway requiring the introduction of molecular oxygen into the substrate [191]. This was confirmed and extended to an imino system in studies of the conversion of the 1,4-benzodiazepinone (23, R = H) to the 3-hydroxy derivative (23, R = OH) [192,193].

A study of the acetic anhydride induced rearrangement of the diazepam-N-4-oxide (24) to the acetyl compound (23, R = OCOMe) was made using highly enriched ^{18}O-labelled acetic anhydride as a model for the possible role of N-oxides as an enzymatic intermediate in microsomal oxidative dealkylation of tertiary amines. The results established that in the system examined, an intramolecular migration of oxygen from nitrogen to carbon can be effected via an activated *N*-acetoxyimmonium intermediate of type (23, R = OCOMe) [193, 194].

Homovanillic acid (25), which is synthesized from dopamine in rat brain, has been labelled with oxygen-18 *in vivo*. The technique has been used to study changes in brain dopamine metabolism following chloropromazine treatment [195,196]. Cerebral oxygen metabolism has been studied *in vivo* in different behavioural situations using gas mixtures of oxygen-18 with natural nitrogen [195]. A special air-tight operant conditioning (Skinner) box has been described that enables oxygen metabolism of rats to be measured using oxygen-18 [197].



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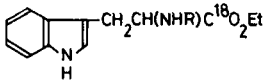

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The rate of bicarbonate exchange across red cell membranes using rat erythrocytes has been measured with the aid of ^{18}O-labelled bicarbonate [198].

When the mechanism of the hydrolysis of sucrose by a sucrase-isomaltase

enzyme from rabbit small intestine was studied in ^{18}O-labelled water it was shown that it was the bond between the glucosyl C-1 and the glycosyl oxygen that was split [199]. The oxygen-18 labelled esters (26, R = COMe or COOMe) have been used to make kinetic isotope measurements of their chymotrypsin catalysed hydrolysis [200].

Variations in natural isotopic abundance have also been utilised. Glucose metabolism was studied in man by measuring the marked change in isotopic ratio (^{13}C : ^{12}C) in expired carbon dioxide after oral glucose administration. The detection of the small differences in ratio is made possible by the great sensitivity of double collector mass spectrometers [201,202]. A more detailed discussion of the use of $^{13}CO_2$ breath tests appears in a later section.

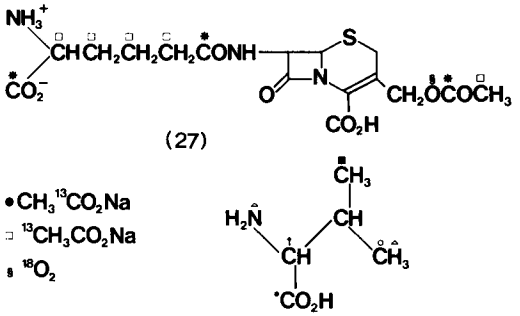
The use of mass spectrometry in conjunction with stable isotopes in the study of detoxification mechanisms has been reviewed [203].

STABLE ISOTOPES IN THE STUDY OF BIOSYNTHETIC PATHWAYS

The use of stable isotopes in the elucidation of pathways of biosynthesis is characterised by two particular features. In many cases, NMR, and in particular ^{13}C-NMR, has been the method of isotope detection employed. Furthermore, in many cases the use of radioactive isotopes and their ease of detection has been combined with the structural definition achievable by the use of stable isotopes in conjunction with NMR. Although much of the reported work has utilised carbon-13 in combination with carbon-14 labelling, there are, nevertheless many instances of the use of nitrogen-15 and oxygen-18 in the study of biosynthetic pathways.

The most rewarding procedure has involved the feeding of specifically ^{14}C-labelled possible precursors to an appropriate culture followed by isolation of the metabolite(s). Screening of the product for radioactivity affords a preliminary indication of the precursors. Subsequent repetition with appropriately ^{13}C-labelled precursors and examination of the product by NMR provides data on the exact location in which the precursors have been incorporated without the necessity for degradative studies. This information is adequate in many cases to deduce the metabolic pathway. In carbon-13 studies, both proton NMR using the satellite method, and ^{13}C-NMR have been employed.

Whilst there are a number of advantages in using a radioactive label in preliminary experiments to establish correct experimental conditions prior to the use of the more expensive stable isotope label, it is not essential. Indeed, in the case of nitrogen15 and oxygen-18 studies, the absence of a suitable radioisotope rules out such a procedure. Furthermore, mass spectrometry may also



*Scheme 1.4. Some studies on the biosynthesis of cephalosporin C. Labelled compounds are indicated as follows: *, sodium [1-^{13}C]acetate [209,210]; □, sodium [2-^{13}C]acetate [209, 210]; •, DL-[1-^{13}C]valine [210]; †, DL-[2-^{13}C]valine [210]; ▪, (2RS,3R)-[4-^{13}C]valine [211]; ○, (2S,3S)-[4-^{13}C]valine [212]; △, (2S,3S)-[^{15}N-(3-methyl-D$_3$)]valine [213]; §, oxygen-18 [214]*

be employed for detection, either with or without the necessity for chemical degradation.

Several useful reviews have appeared on the use of stable isotopes in biosynthetic studies [122,123,125,126,203–206], and on the instrumental methods employed [207]. The techniques will be illustrated in this section by a number of examples.

Perhaps the most widely studied area, and at the same time the area of most interest to the medicinal chemist, is that of antibiotic biosynthesis. The widespread interest is no doubt related to the high incorporation of carbon-13 into bacterial or fungal metabolites and because of the high isolated yields.

The technique of carbon-13 labelling in the study of antibiotic biosynthesis utilising NMR detection methods has been reviewed with specific reference to the β-lactam antibiotics [204]. The biosynthesis of penicillins and cephalosporins has recently been reviewed and stable isotopes have played a significant role in these studies [208].

The incorporation of ^{13}C and ^{15}N stable isotope labelled precursors into the molecule of cephalosporin C (27) is illustrated in *Scheme 1.4.* The reader is referred to the original papers for the detailed procedures and discussion of the results. Information on the incorporation of [^{13}C]acetate and various [^{13}C]-valines was obtained from ^{13}C-NMR studies of cephalosporin C after incubation of the culture with the appropriately labelled precursor [204,209–212]. Most of the side chain carbon atoms where shown to be derived from acetate residues by feeding sodium [1-^{13}C]acetate and sodium [2-^{13}C]acetate [209,210]. The incorporation of the entire valine skeleton into both penicillin N and cephalosporin C was shown by the use of (2*S*,3*S*)-[^{15}N-(3-*methyl*-D$_3$)]valine [213]. The

findings supported the suggestion that both the β-lactam antibiotics originate from a common tripeptide intermediate such as δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine [213]. When mycelial suspensions of *Cephalosporium acremonium* were shaken in an oxygen atmosphere enriched in oxygen-18, and the cephalosporin C isolated, it was shown that one atom had been incorporated at C-17 (see *Scheme 1.4*) providing direct evidence of the participation of hydroxylase in the biosynthesis of this antibiotic [214].

An interesting use of mixed radioisotope and stable isotope labelling was demonstrated when the formation of benzylpenicillin by *Penicillium chrysogenum* in the presence of L- and D-[3-^{14}C,^{15}N,^{35}S]cystine was studied. The penicillin isolated contained the three labels in their proportions in the precursor when L-cystine had been added to the fermentation medium. It was concluded that cystine was a direct precursor of penicillin, probably after reduction to cysteine [215].

The ansamycin antibiotics have also received a substantial amount of attention, particularly because of their wide spectrum of activity. Much of this work has been reviewed [125,205,206].

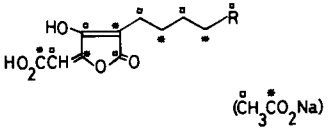
Although the use of ^{14}C-labelled acetates and propionates had demonstrated that they were precursors in the formation of rifamycin S it was only by the use of various ^{13}C-precursors and assignments of the NMR spectra that the labelling pattern from acetate and propionate was established [216]. ^{13}C-NMR has also been used to establish the biosynthetic pathway of streptovaricin D; the use of sodium [1-^{13}C]propionate established that the amide carbonyl was derived from the carboxyl group of propionate [217]. The use of methionine (^{13}C-methyl) and sodium [1-^{13}C]propionate showed that geldanamycin followed the same biosynthetic pathway as rifamycin and streptovaricin D [218].

Lasalocid A, an antibiotic produced by *Streptomyces lasaliensis*, is unique in that it is a polyether antibiotic and that it contains 3 C-ethyl groups. Studies on the incorporation of sodium [1-^{13}C]acetate, sodium [1-^{13}C]propionate, and sodium [1-^{13}C]butyrate, and examination of the labelled antibiotic with ^{13}C-NMR, confirmed that the lasalocid A skeleton was derived from 5 acetate, 4 propionate, and 3 butyrate residues. This work provided the first example of C-ethyl groups arising from complete butyrate units. Indeed, all 3 C-ethyl groups arose in this way [219,220].

[6-^{13}C]Glucose has been employed in work on the biosynthesis of streptomycin [221] and neomycin [222], while the incorporation of [1-^{13}C]glucosamine into neomycin has also been studied [222].

Studies with [1-^{13}C]acetate have demonstrated that acetate is the source of the exterior carbons of the pyridine ring of nybomycin but not of the central ring [223].

The biosynthetic proposals for the origin of the macrocyclic mould metabo-



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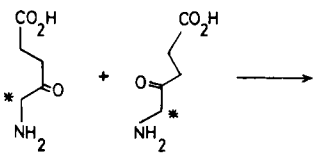

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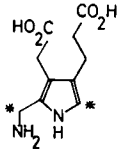

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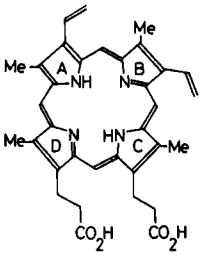

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lites cytochalasin B (phomin) and cytochalasin D (zygosporin A) have been corroborated. Incorporation of sodium [2-^{13}C]acetate into the former, and sodium [1-^{13}C]acetate and sodium [2-^{13}C]acetate into the latter, was studied by ^{13}C-NMR; rates of incorporation were determined by mass spectrometry [224].

Other examples of the use of acetate-^{13}C in the elucidation of structure and biosynthesis include multicolic acid (28, R = CH_2OH) and multicolosic acid (28, R = CO_2H) isolated from cultures of *P. multicolor* using [1-^{13}C]^2-, [2-^{13}C]-, and [1,2-^{13}C]acetate [225], tajixanthone, a metabolite of *Aspergillus variecolor* using [1-^{13}C]- and [2-^{13}C]acetate [226], and aflatoxin B_1 from *A. flavus* using [2-^{13}C]- and [1,2-^{13}C]acetate [227]. The study of the metabolites of *P. multicolor* utilised a unique method for the detection of intact acetate residues. After feeding sodium [1,2-^{13}C]acetate, the presence of such residues in the metabolites produced were detected by the presence of ^{13}C–^{13}C coupled satellites superimposed on natural abundance singlets in the proton decoupled ^{13}C-NMR spectrum. It provided the first example of the use of doubly labelled acetate to establish an intermediate aromatic precursor in the biosynthesis of a fungal metabolite [225].

Elegant work based on double ^{13}C-labelling and NMR spectroscopy has demonstrated the process by which natural type III porphyrins are biosynthesized [228,229]. [2,11-$^{13}C_2$]Porphobilinogen (29) was prepared enzymatically from amino [5-^{13}C]laevulinic acid (30), the preparation of which had previously

been described [230]. It was diluted with 4 parts of unlabelled material, converted by a coupled enzyme system from chicken blood cells and beef liver mitochondria into the dimethyl ester of protoporphyrin-IX (31), and examined by ^{13}C-NMR spectroscopy. The biosynthesis was characterised by the fact that 3 porphobilinogen units were incorporated intact to give the A, B and C rings with their attendant bridges. The porphobilinogen unit that formed ring D was built in with an intramolecular rearrangement; the rearranged carbon atoms formed the bridge at C-15 (linking rings C and D).

Extensive work has been reported on ^{13}C-NMR studies of ^{13}C-enriched samples of vitamin B_{12} [231]. The enrichment was carried out by feeding 5-amino [2-^{13}C]laevulinic acid, 5-amino [5-^{13}C]laevulinic acid, [8-^{13}C]porphobilinogen, L-methionine (^{13}C-methyl), and ^{13}C-labelled uroporphyrinogen. The study, which provided evidence of the location of the labelled centres in the vitamin, showed the intact incorporation of porphobilinogen and uroporphyrinogen III into vitamin B_{12}.

However, NMR has not been the only method of detection that has been used in biosynthetic studies. Indeed, for oxygen-18, mass spectral studies have been essential and have also been employed with deuterium, carbon-13, and nitrogen-15. Much of the work has been reviewed [203]. Studies on the biosynthesis of steroids have been particularly fruitful. Atmospheres of oxygen-18 have been used to study side chain cleavage of 17-α-hydroxyprogesterone and of cholesterol. The incorporation of oxygen in the formation of prostaglandin E_1 and many other studies of biochemical interest have also been reviewed [203]. Mass spectrometry is clearly a useful method of studying products that are formed biochemically in the presence of oxygen which can readily be replaced by oxygen-18.

CLINICAL APPLICATIONS OF STABLE ISOTOPES

The following sections are intended to present, in general terms, an appraisal of the scope and depth of the involvement of stable isotopes in quantitative clinical investigations. In the majority of investigations isotope mass spectrometry has been the analytical system of choice. It is not our intention to present a critical review of experimental protocol or data handling, but primarily to indicate areas of research where stable isotopes have played, and will continue to play, an important role in clinical studies. A chronological development of certain themes seems pertinent in many instances as much clinically orientated isotope investigation has stemmed from work initiated during the decade 1935–1945.

The literature cited covers isotope dilution studies, such as the use of deute-

rium oxide for estimating total body water, and a range of metabolically defined problems. In this latter category one would stress the development of ^{13}C breath tests in the 'diagnostic' field and a diversity of studies using ^{15}N-labelled precursors to monitor specific areas of nitrogen metabolism in man in both normal and diseased states.

DEUTERIUM

Since the discovery of the heavy stable isotope of hydrogen in 1931, deuterium oxide has been used extensively in biological and clinical investigations. It constitutes an ideal tracer both for the determination of total body water and also the study of the dynamics of water distribution and compartmental water exchange in the human subject.

The large percentage mass difference between the two hydrogen isotopes resulting in the deuterium 'isotope effect' repeatedly demonstrated at both enzyme [232] and whole body level [233] when employing deuterium at high concentrations, need not be amplified at this point. The oral or intravenous administration of 200 g D_2O to an adult human male subject will increase the plasma deuterium concentration to approximately 0.5%. Natural levels of deuterium oxide in plasma range between 0.0145 and 0.0155% depending on diet and source of drinking water. To date, few untoward effects of deuterium oxide administration to human subjects at the 200 g level have been reported. In five subjects studied [234], vertigo occurred some 30 min after ingestion of deuterium oxide and in another report [235] nystagmus (involuntary oscillating movement of the eyeballs) occurred in all 10 male subjects investigated. There has been an isolated account of a transitory increase in a gradually developing presbyopia [234].

Total body water – variation with age

The accuracy with which total body water can be measured depends on the uniformity of distribution of the deterium oxide throughout the water compartments of the body and the precision with which the concentration of deuterium can be measured in a given body fluid. As early as 1959 it was demonstrated that salivary glands concentrated deuterium above the level in serum, following the administration of label to normal pregnant women [236]. This fact has been repeatedly questioned by various workers [237] on the grounds of possible contamination of the salivary distillate, required for deuterium analysis by the 'falling drop' method employed. More recently the use of isotope mass spectrometry has provided unequivocal evidence that in both the rat [238] and man

[239,240], urine and the aqueous phase of the plasma are differentially enriched in deuterium with respect to the drinking water normally consumed. A practical implication of these findings is that it is necessary to compare the deuterium content of the same body fluid before and after the administration of label, if accurate estimates of body water are to be obtained.

A variety of analytical methods have been employed for the estimation of deuterium in biological fluids in the context of total body water measurements. These include the falling drop method [241], freezing point elevation [242], infrared spectroscopy [243], gas chromatography [237] and mass spectrometry [240,244]. A range of accuracy between ±0.5–5.0% is claimed from these various techniques in the overall protocol of body water measurement.

The introduction of deuterium oxide into the body results in the exchange with H_2O molecules to form HDO. In addition D atoms will exchange with labile hydrogen atoms of carboxyl, hydroxyl, amino, imino and sulphydryl groups of organic molecules. Exchange does not normally occur with hydrogen atoms bound directly to carbon [245]. The result of these exchanges will be an apparent increase in the 'volume of dilution' of the administered tracer, a lowering of the equilibrated deuterium concentration and hence an overestimate of total body water. It has previously been estimated that this error will represent a water equivalent of 0.5–2.0% of body weight [246]. More recently a comprehensive treatment of a theoretical calculation of the total non-aqueous exchangeable hydrogen in protein, fat and carbohydrate in man has been presented [247]. It was demonstrated that 5.2% of total exchangeable hydrogen in the body is accounted for by water-soluble components. This represents the maximum possible overestimate of total body water using deuterium oxide. It is apparent from comparative isotope dilution–desiccation studies that the 2–4 h period of equilibration in total body water measurements does not permit complete exchange to occur. It is suggested that the slowly exchanging protein amide hydrogens may account for the discrepancy [248].

Validation of body water measurements, using dilution principles and either of the hydrogen isotopes, can only be achieved from complementary desiccation studies. In fact it has been repeatedly demonstrated in animals that the volume of dilution of deuterium oxide corresponds closely to the total body water space [249–252]. A single report has suggested that the difference between isotope dilution using tritium and desiccation, was statistically significant in the rat and that the former method overestimated body water by 12% of the desiccation value [253].

As a generalisation, it is true to state that, when water content is expressed as a percentage of body weight, a continual dehydration of the body occurs from developing foetus to late adulthood. At a gestational age of one month, the foetus is more than 90% water, but at birth published figures estimate a water

Table 1.2. VARIATION OF TOTAL BODY WATER WITH AGE, EXPRESSED AS A PERCENTAGE OF BODY WEIGHT, IN HUMAN SUBJECTS. MEASUREMENTS FROM ISOTOPE DILUTION STUDIES

Age	*Sex*	*No. of subjects*	*Condition*	*Body water as % body weight*	*Reference*
1 d	M	1	Full term	78.6	255
1 d	M	1	Full term	79.6	257 [a]
3 d	F	1	Full term	78.8	255
4 m	M	1	Post meningitis	70.7	255
18–32 y	M	17	Normal	61.8 ± 3.5	259
18–31 y	M	20	Normal	64.0 ± 3.2	– [b]
20–31 y	F	11	Normal	51.9 ± 4.7	259
21–33 y	F	20	Normal	53.5 ± 3.5	– [b]
12–59 y	F	27	Obese	40.3 (range 27.1–65.2)	262
46 y	M	1	Skull fracture	55.1	263 [a]

[a] Cadaver desiccation.
[b] D. Halliday (unpublished data).

content of 73–79% [254–256]. These estimates are in fairly close agreement with results obtained by desiccation of full term cadavers [247,258]. There is a gradual relative loss of water during the first 6 months of life and thereafter in early childhood values ranging between 53 and 63% have been presented that show no correlation between age and sex though correlations exist between body weight and surface area and the actual values of body water expressed as litres [255]. Sex differences are manifest in adults as demonstrated by results obtained by isotope dilution and mass spectrometric analysis of deuterium [259]. The differences in water content undoubtedly result from the relatively greater lean body mass (muscle) in the male, of high water content [260] and the corresponding greater adipose tissue mass, of low water content [261], in the female subject. These differences are exaggerated in trained male athletes whose body water may account for 72% body weight, whilst in grossly obese women water may represent less than 30% of body weight. A summary of total body water estimates obtained by isotope dilution and desiccation at various ages are presented in *Table 1.2.*

Body water changes during pregnancy

An increase in body water accounts for a large percentage of weight gain exhibited during a natural pregnancy. This occurs as a result of placental and foetal development, the formation of amniotic fluid and an increased maternal plasma

volume. The development of general or localised oedema is not uncommon during pregnancy, due to an increase in the extracellular water space. Deuterium, lacking radiation hazard, is obviously the tracer of choice in this instance, not only to monitor serial changes in total body water during pregnancy and post-partum, but also to investigate maternal–foetal–amniotic water transfer.

Three groups of investigators have used deuterium oxide to follow the reduction in maternal total body water from late pregnancy to post-partum [264–266]. Mean water loss was approximately 6 l though the range was large (0–14.2 l) and the number of women studied was few.

In a more recent comprehensive study involving 93 women, total body water was estimated on four occasions during pregnancy and also post-natally [267]. The mean water gain during pregnancy was found to be 8.5 kg. Women exhibiting no oedema gained least, those with generalised oedema most and those with localised leg oedema gained an intermediate amount.

The exchange of water within the tripartite system of developing foetus and maternal and amniotic fluids has been investigated with deuterium oxide alone or in conjunction with tritium. Variation in the rate of transfer of water to the foetus and placenta (per unit weight) with respect to gestational age have been monitored [268]. In both cases a 5-fold increase in water transfer was observed between 14 and 35 wk gestation with a rapid decrease before term. An alternative approach using both hydrogen isotopes injected into the amniotic sac prior to delivery, has provided further information [269]. In early pregnancy, water exchange between amniotic fluid and mother is quantitatively equivalent to the mother to foetus exchange. In late pregnancy however the foetus assumes a dominant role in this transfer such that some 40% of water transfer from amniotic fluid to mother is through the foetus. It was calculated that at term, the water exchange between mother and foetus is of the order of 3.5 l/h.

Body composition and energy balance

To the clinician, the accurate measurement of total body water (TBW) can provide indirect information concerning whole body composition and energy balance. Desiccation measurements performed on eviscerated guinea pigs in 1945 [270] led to the almost universal acceptance of the fact that lean body mass (LBM) was of constant composition – and additionally, contained all of the body water. Thus the extracellular water associated with adipose tissue was neglected and the water content of lean body mass taken as 73.2%. The following simple formula, which was readily applied to man, provided an indirect estimate of body fat:

$$\text{Fat(kg)} = \text{Body weight(kg)} - \frac{\text{TBW(kg)}}{0.732}$$

Credance was given to this formula from 6 whole body chemical analyses performed on adult cadavers where the mean water content of LBM was 72.5% (67.4–77.5%) [271]. That the use of this formula, even for subjects of normal body composition, may be an over-simplification leading to significant errors has been stressed [272]. Certainly it is inapplicable to individuals exhibiting oedema, malnutrition, obesity or during pregnancy. The study of obesity with respect to body composition, has prompted the development of a new model for calculating adipose tissue and adipose-free masses [261]. The model still requires the measurement of total body water and its application to 4 normal and 5 obese individuals has been presented [273].

The control of energy balance in man is obscure and the study of energy balance is complicated by the fact that alterations in body weight may reflect a change in energy stores or an alteration of total body water, or both. As direct methods for the estimation of body fat or protein in human subjects are lacking the state of hydration assumes importance in energy balance studies. The total body water of an adult male is approximately 40 kg and the energy value of adipose tissue is some 7000 kcal (29 MJ) per kg. Thus if total body water can be measured to ±200 g (0.5%) then the error incurred in measuring energy balance is only 1400 kcal (5.8 MJ).

Intercompartmental water movement

The rate of water exchange between discrete body fluid compartments can be estimated from the rate of appearance or disappearance of deuterium oxide into or from a given compartment. Two possibilities exist; the intravenous injection of deuterium oxide and thence monitoring its rate of appearance in the water compartment under investigation, or a direct reversal of this procedure. If, for instance, the label is injected into a synovial capsule and exchange with the blood occurs at a constant rate, then an exponential decay curve of isotope concentration within the capsule may be constructed. This of course assumes no net transfer of water over the measurement period. The exponential decay constant will reflect both the fluid volume within the capsule and the capsule–blood water exchange rate (per unit time). Decay rate constants have been determined for the aqueous and vitreous compartments of the eye in the rabbit and monkey [274] and for cerebrospinal fluid in man [275]. The time required to reduce the deuterium concentration by one-half ($t_{1/2}$) for cerebrospinal fluid was found to be 1.5, 8–11 and 18–26 min at the cisternae, ventricular and lumbar regions respectively. These investigations were performed during craniotomies and involved the additional use of radioactively labelled sodium chloride. The use of both isotopes permitted definitive statements to be presented regarding the formation of cerebrospinal fluid. This work was later confirmed when

deuterium oxide was instilled into the lateral ventricles of patients with hydrocephalus and shown to equilibrate rapidly with cerebrospinal fluid, via the blood, in the lumbar region [276].

Water uptake from the stomach has been investigated using deuterium oxide [277]. In eight normal subjects the disappearance rate of administered label was found to be 2.5% per min giving 95% disappearance at approximately 54 min. In a separate study [278], it was calculated that the net absorption of water from the stomach amounts to 230 ml per h. It was also demonstrated that this water movement is somewhat reduced in patients exhibiting gastric atrophy.

In terms of the whole body, the dynamics of distribution of deuterium oxide between blood and extravascular water have been determined following the intravenous injection of the label [259]. The plasma pre-equilibrium concentration curve plotted semi-logarithmically with respect to time was composed of 2 exponentials, described by the equation:-

$$C_t = Ae^{-\lambda_1 t} + Be^{-\lambda_2 t} + C_{eq}$$

where C_t is the concentration of deuterium in arterial serum at time t, C_{eq} is the concentration in arterial serum at equilibrium; A and B are constants and λ_1 and λ_2 are rate constants for the two components of the curve. The half-lives of these two components were calculated to be 1.25 and 9.5 min. Whilst these two curves undoubtedly represent an oversimplification of isotope exchange between intra- and extra-vascular compartments, it has been suggested that they may reflect groups of 'fast' and 'slow' capillaries [279], or alternatively differential rates of transmembrane water movement [259]. Additional information resulting from these studies has demonstrated that the half life of deuterium in a normal adult is 9.3 ± 1.5 days.

Finally in this broad area of study two isolated and unrelated applications of deuterium oxide may be briefly mentioned. Haemodialysis constitutes the normal treatment used as a prelude to renal transplantation. Peritoneal dialysis, however, provides a method for alleviating temporary renal failure. Deuterium oxide has been used to determine the kinetics of water equilibration between the body water and dialysis fluid in an attempt to improve the efficiency of peritoneal dialysis [280].

Deuterium has also been employed to investigate the quantitative movement of water through the skin as this may have some bearing on the environmental conditions under which certain groups of patients are maintained [281]. An extension of this type of study is the potential use of deuterium oxide or deuterated pharmaceuticals to study the absorption efficiency of percutaneous drug administration.

Biochemical investigations

In estimating total body water by isotope dilution using deuterium oxide, an error (an overestimate) arises if the exchange with labile hydrogen atoms attached to atoms other than carbon is ignored. In addition to this labile exchange during equilibration, deuterium will be incorporated into the molecular skeleton of organic species as normal synthesis proceeds, whilst unlabelled hydrogen will be returned to the 'pool' as a result of the oxidation of fat and carbohydrate. These synthetic and catabolic processes would, however, contribute an insignificant error in measuring total body water, over the time period required for the equilibration of administered deuterium oxide. The potential use of these latter reactions received much attention in the late 1930's for estimating the synthesis and degradation rates of fatty acids in animals [282], whose body water was maintained at an elevated equilibrium level of deuterium for a considerable period. Plasma plateau labelling is normally achieved with a priming dose of deuterium oxide followed by daily supplements to maintain the equilibrium level in the face of water intake. This protocol has been applied to human subjects to estimate the rate of serum cholesterol synthesis [234, 283]. In both reports, similar results were obtained ($t_{1/2}$ = 8–12 days). In the earlier study the level of enrichment attained in the serum cholesterol was lower than that subsequently found. The discrepancy probably results from the fact that in the first report a single subject was on a relatively cholesterol-rich diet and that the actual deuterium estimations were performed on the digitonide derivative. Both these events would act to depress the level of deuterium as measured in the cholesterol. Using this experimental technique and feeding either high cholesterol or cholesterol-free diets, it was possible to confirm earlier work in demonstrating an apparent lack of any feedback control of endogenous serum cholesterol synthesis [284]. This experimental approach is of course not limited to cholesterol metabolism but may be applied to human subjects to study fatty acid metabolism in a manner analogous to that used in rats with tritium label [285].

The use of deuterated pharmaceuticals to study intermediary drug metabolism with GC–MS analytical techniques has increased enormously in the last decade and has been discussed in a previous section. Quantitative isotope mass spectrometry may well come into its own in this field in the near future for general screening. Whilst individual metabolites cannot be monitored, the total deuterium excreted in the urine can be accurately measured and thus provide a good indication of the excretion time course of total deuterium label.

CARBON-13

Breath tests

In recent years the development of $^{13}CO_2$ breath tests have provided the clinician with another way to employ stable isotopes in diagnosis. The rationale behind these tests is that a patient presenting a metabolic disorder may oxidise a specific ^{13}C-labelled substrate more slowly or more rapidly than a normal subject. Thus the $^{13}CO_2$ breath test relies on the choice of a specific ^{13}C-labelled substrate where the cleavage of a 'target' bond results in the release of a functional group destined to produce $^{13}CO_2$ as a metabolic end product. The appearance of an excess of $^{13}CO_2$ in exhaled air provides an indication of both the rate and extent to which the 'target' bond has been acted upon enzymatically. Obviously this type of test can only provide an overall assessment of the particular metabolic disorder being investigated. An excess of $^{13}CO_2$ may appear in exhaled air as a result of the oxidation of acetate in the tricarboxylic acid cycle, the oxidative decarboxylation of pyruvate or the oxidation of the first carbon of glucose-6-phosphate in the pentose shunt. In addition, the chosen substrate may be subjected to initial hepatic transformation and, if the label is administered orally, absorption rates must be considered. Methods for the collection of $^{13}CO_2$ in exhaled air for mass spectrometric isotopic analysis have been previously presented (p. 26). *Table 1.3* lists some of the specific $^{13}CO_2$ breath tests of diagnostic interest that have been applied to human subjects. Theoretical and practical implications of breath tests involving the use of ^{14}C-labelled substrates have been comprehensively reviewed with respect to various metabolic disorders [286].

To date, the development of breath tests has evolved around either glucose tolerance tests in obesity and the diabetic state, or the investigation of liver

Table 1.3. CLINICAL APPLICATIONS OF THE $^{13}CO_2$ BREATH TEST

Substrate	*Application*	*Reference*
[$^{13}C_2$]Dimethylaminopyrine	Hepatic microsomal function	290
[U-^{13}C]Galactose	Alcoholic cirrhosis	293
[U-^{13}C]Glucose (natural abundance)	Normal, diabetes	201,301
[U-^{13}C]Glucose	Diabetes (children)	294
[$^{13}C_2$]Glycocholate	Bacterial overgrowth (small intestine)	292
[$^{13}C_3$]Trioctanoin	Fat malabsorption	287

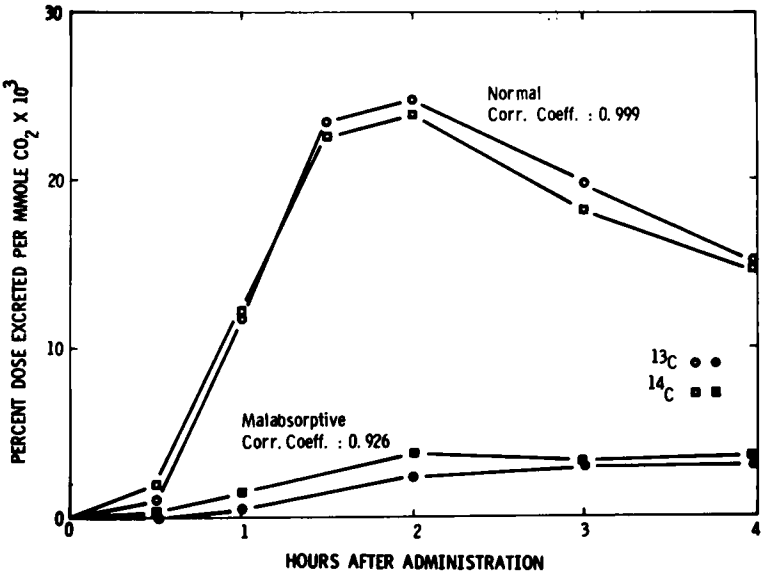

Figure 1.7. Direct comparison of the ^{13}C and ^{14}C content of expired air following the simultaneous administration (oral) of [^{13}C]- and [^{14}C]trioctanoin, Normal subject: ○,□; malabsorption patient: ●,■

and gastrointestinal disorders. Of interest in the latter category is the development of a sensitive test for fat malabsorption which has been applied to 9 children between the age of 3 months and 5 years [287]. [^{13}C]Trioctanoin, a medium chain fatty acid, was administered orally, and the exhalation of $^{13}CO_2$ monitored for 4 h. (*Carboxyl*-^{13}C)-labelled trioctanoin absorption in the gastrointestinal tract leads to the rapid appearance of $^{13}CO_2$ in exhaled breath. The presence of malabsorption, resulting in the excretion of administered label into the colon, is manifest by a marked diminution in the production rate of $^{13}CO_2$. In control children some 25% of the label was excreted in 2 h, whilst in children exhibiting fat malabsorption (cystic fibrosis and steatorrhea) only 3.5% of the label was excreted. The authors stress the specific advantages of this test as applied to a pediatric population for both mass screening and diagnostic potential. *Figure 1.7* shows a comparison of the excretion of $^{13}CO_2$ and $^{14}CO_2$ after simultaneous oral administration of both [^{13}C]- and [^{14}C]-trioctanoin. Routine adoption of this malabsorption test would preclude the necessity of the more conventional 3–4 days stool collection and analysis following a fat load, requiring in-patient supervision in a metabolic ward.

Studies of an analogous nature to the ^{13}C-labelled trioctanoin breath test have

been reported and are all of clinical interest. The metabolism of chemotherapeutic agents by the liver and their effect on either induction or inhibition of the hepatic microsomal mixed function oxidase enzyme system have been investigated in man using [^{14}C]dimethylaminopyrine (DAP) [288,289]. These tests have now been extended to be applicable to a wider range of the population with the use of [^{13}C]DAP [290]. The oral administration of [^{13}C]DAP to 4 normals and 5 patients with cholestatic liver disease followed by half-hourly $^{13}CO_2$ breath analysis, demonstrated the rate of N-demethylation of [^{13}C]DAP and provided information regarding the hepatic microsomal functional mass. The administration of phenobarbitone increased the production of $^{13}CO_2$ in both normal and diseased subjects which is in agreement with the findings from equivalent radioactive studies [288].

The rate of clearance of a galactose load from the circulation provides a sensitive indication of possible hepatic dysfunction and readily discriminates between cirrhotic and non-cirrhotic subjects [291]. Estimations of the rate of oxidation of an orally administered galactose load to expired $^{13}CO_2$ using a ^{13}C label provide diagnostic information analogous to that obtained from galactose clearance studies. Initial investigations using both [^{14}C]- and [^{13}C]galactose [92] have been reported showing that the mean rates of expiration of labelled CO_2 by the patients with cirrhosis were one-third to one-half of mean normal rates, during the first 90 min, following galactose administration. Further it was found that peak labelling of expired $^{13}CO_2$ was achieved one hour later in cirrhotic patients compared with normals.

Another study, recently performed, has compared the results obtained in terms of bacterial overgrowth or ileal dysfunction in adults presenting a variety of gastrointestinal diseases using both [^{14}C]- and [^{13}C]glycocholate [292]. $^{14}CO_2$ and $^{13}CO_2$ measured by scintillation counting and mass spectrometry respectively, were evolved in direct proportion ($r = 0.962$), and deconjugation of 5–10% of the administered glycocholate was demonstrated. In a child with an ileal bypass of some 100 cm, almost complete deconjugation was found. Admittedly the ^{13}C-labelled substrates used in the above investigations are not readily available at present, but it will only be by clinical demand for these diagnostic tests that commercial organisations may be persuaded to undertake the necessary syntheses.

The development of the [^{13}C]glucose tolerance breath tests probably presents far greater problems in terms of interpretation of results than the examples cited above where use is made of a single substrate to investigate a specific metabolic area. Thus, variation of the rate of appearance of $^{13}CO_2$ in exhaled air following a [^{13}C]glucose load will represent the combined effects of absorption, the formation of trioses, the decarboxylation of pyruvate, oxidation within the pentose shunt or the effect of gluconeogenesis. Various workers have shown that

the rate of oxidation of ^{13}C-labelled glucose to $^{13}CO_2$, when administered with a glucose load, is low in obese patients with variable glucose tolerances, in non-obese, non-ketotic diabetic patients with major glucose intolerance [293] and in untreated diabetic children [294]. In the case of the moderate diabetic adults, the $^{13}CO_2$ data suggest the reduced excretion reflected a failure to reduce gluconeogenesis following a glucose load, rather than a specific impairment of the glucose oxidation system. Corroboration of these findings may be obtained using ^{13}C-labelled pyruvate or lactate [294]. Untreated diabetic children who received a single injection of insulin showed a rapid response in the rate of $^{13}CO_2$ expiration, and 2–3 days of insulin treatment resulted in both the time course and peak values of $^{13}CO_2$ excretion being within the normal range [295]. These authors suggested that the differences observed in $^{13}CO_2$ excretion between normal and diabetic children may be explained in terms of variable pool size and increased production, but decreased oxidation, of glucose. Renal losses of glucose were negligible in the diabetic children studied.

A group of Belgian workers have recently produced a series of papers demonstrating the use of naturally occurring [^{13}C]glucose obtained from maize, for oral glucose tolerance tests. Plants effect photosynthesis either by the Calvin pathway [296] or via the dicarboxylic acid pathway [297]. As a result of the selective isotopic effect [298] plants photosynthesizing by the latter pathway, such as maize or sugar cane, have a $\delta^{13}C$ of 18‰ compared to beet sugar with $\delta^{13}C$ of approximately 4‰. Using this readily available natural label preliminary glucose oxidation estimates were performed in rats [299,300] and the developed methodology applied to human subjects. Initially 6 normal male subjects were used to demonstrate a marked, reproducible rise in $^{13}CO_2$ expired air, which reached a maximum in 4 h, and was still detectable at 8 h [201]. These studies have been extended to diabetic patients, and additional measurements of blood glucose and respiratory quotient permit the comparison with normal subjects of total glucose oxidised and the fraction of exogenous glucose oxidised following the oral load [301]. That the rise in expired $^{13}CO_2$ seen in normal subjects may be due to a reduction in lipid oxidation in addition to the increased utilization of endogenous glucose has been suggested [201]. It has previously been demonstrated in both plants [302] and animals [303] that variations in respiratory $^{13}CO_2$ content can reflect the origin of the primary endogenous substrate undergoing oxidation. The possibility therefore exists that similar measurements may complement respiratory quotient estimates in human subjects exhibiting various metabolic disorders.

The advantages of non-invasive $^{13}CO_2$ breath tests in diagnosis, therapeutic monitoring, mass screening potential and simplicity of protocol are numerous. However, on the debit side, the $^{13}CO_2$ content of respired air fluctuates even in the fasting state with a standard error of some ±0.7‰ [304]. In practical terms

this means that the expired $^{13}CO_2$ must be greater than 1.4% during a 6 h study in order to attain significance at the 95% level. In turn this requires a minimum substrate oxidation rate of 140 nM kg^{-1} h^{-1}, but for the true diagnostic potential, an order of magnitude greater than this is desirable. As outlined above, the major applications of $^{13}CO_2$ breath tests have centred around gastrointestinal and hepatic disorders and glucose metabolism in obese and diabetic patients. It is a matter of conjecture whether further metabolic disorders may be approached in a similar manner in the future.

Glucose metabolism

The commercial availability of [^{13}C]glucose, variously labelled, has permitted the use of this substrate in quantitative estimation of glucose turnover in both the human newborn and in malnourished and hypoglycaemic children. Immediately post-partum, the newborn infant must be in a position to provide its own supply of glucose, be it from hepatic glycogen stores or via gluconeogenesis. The use of a prime-constant-infusion technique [305] with [1-^{13}C]glucose as tracer has permitted quantitation of glucose production in the human newborn [306]. Employing this technique, a steady state of plasma ^{13}C was obtained within 1 h of the infusion and maintained for at least 30 min. The calculated systemic glucose production rate in 2 h infants was 4.4 mg kg^{-1} min^{-1} whilst at 1 day the rate was slightly lower (3.8 mg kg^{-1} min^{-1}). An interesting observation from these studies was the dilution of fasting glucose ^{13}C in the 1 day ($^{13}C = 27.5$) compared to the 2 h old infant ($^{13}C = 16.3$). This may indicate different sources of glucose or possible enzymatic fractionation during glucose metabolism.

In an independent study, glucose flux was measured in 5 severely malnourished infants of 1 to 2 yr old using the primed constant infusion technique with [U-^{13}C]glucose as tracer [307]. Glucose flux was estimated at 3.0 mg kg^{-1} min^{-1} (range 1.8–4.4) in the malnourished state and not significantly different in the same infants after recovery, 2.7 mg kg^{-1} min^{-1} (range 2.4–3.2).

Miscellaneous

The potential use of ^{13}C-labelled compounds in clinical research has not yet been fully appreciated. There is an increasing demand for ^{13}C-labelled amino acids to complement ^{15}N studies of human nitrogen metabolism in addition to ^{13}C fatty acids, which would permit the study of fatty acid and triglyceride metabolism to be extended to patients in whom the use of ^{14}C analogues are ethically unacceptable. One of the few reports involving the use of ^{13}C-labelled fatty acids employed palmitic and oleic acids to study fat absorption in a patient with chyluria [308]. These investigators fed (*carboxyl*-^{13}C)-labelled

palmitic or oleic acids as free acid or triglyceride ester to the patient and followed their incorporation into different lipid groups of the lymph. The absorption of these fatty acids was almost complete in all studies and comparison of the ^{13}C content of administered acids and the extracted lymph lipid fatty acids indicated that approximately 90% of the chyle triglycerides were of dietary origin. Whilst this particular study was made possible by the presentation of a rare clinical condition, it points the way for future studies on fatty acid metabolism in human subjects. For instance, it has been recognised since the early 1930's that pregnancy is accompanied by an increase in the lipid fractions of the plasma [309] and more recently, elevation of plasma cholesterol, triglycerides, phospholipids and free fatty acids has been confirmed [310–312]. The application of experimental procedures, involving the constant infusion of label, designed for use with ^{14}C tracers [313] could be applied to patients pre- and post-partum to monitor plasma free fatty acid and triglyceride transport in pregnancy.

NITROGEN-15

Urea and ammonia

It was first demonstrated in the rat that urea nitrogen could be utilized as a non-essential nitrogen source for protein synthesis, given an adequate supply of essential amino acids, in the absence of additional exogenous nitrogen [314]. Within 3 yr, it was reported that approximately 20% of the urea synthesized by normal man is degraded in the gastrointestinal tract [315]. This hydrolysis to ammonia is effected by ureases from the intestinal flora [316] whose action may be inhibited by the administration of broad-spectrum antibiotics [317]. In health, 3–4 g per day of ammonia nitrogen are derived from hydrolysed urea and may be utilized for protein synthesis [315,318].

The ammonia enters the portal vein and mixes with the metabolic nitrogen pool of the body. The fixation of this ammonia nitrogen into arginine, glutamic acid and glutamine and aspartic acid represents a net contribution to nitrogen balance. The alternative fate of this ammonia is its conversion to urea prior to renal excretion. Thus the use of ^{15}N-labelled ammonium salts or [^{15}N]urea forms the basis of several common lines of clinical investigation.

[^{15}N]Urea was used to maintain a normal growth rate in protein-deprived infants and the label was shown to be present in both haemoglobin and plasma protein [319]. In an analogous situation of protein depletion, uraemic patients had improved nitrogen balance when fed only urea and 2 g of essential amino acid nitrogen [320]. This group later demonstrated the incorporation of [^{15}N]-

urea into amino acids of patients with chronic renal failure who were maintained on low nitrogen diets [321]. It is in the study of specific areas of nitrogen metabolism, in malnourished children and uraemic patients that [^{15}N]urea and ammonia have been most widely employed.

Early studies with [^{15}N]ammonia in animals [322,323] and later in uraemic and normal adults [324] have shown that restriction of dietary nitrogen intake results in a greater retention of [^{15}N]ammonia than in the corresponding control subject. In healthy individuals, the more severe the protein restriction, the greater was the incorporation of [^{15}N]ammonia into plasma albumin, with a correspondingly smaller recovery of label in urine and faeces. In comparison, two 9 month protein-restricted stable uraemic patients incorporated 3–5 times as much [^{15}N]ammonia into plasma albumin as did healthy individuals after 3 wk on a low protein diet. These authors calculated from their results that incorporation of [^{15}N]ammonia into plasma albumin had a theoretical protein equivalent of 4.5–6 g [324]. They further indicated that uraemic patients might increase the reutilization of urea nitrogen if provided with dietary keto-acid analogues of essential amino acids, with the exclusion of other exogenous nitrogen, (except lysine and threonine). This possibility had been alluded to previously [325]. Experimental support for this concept was later obtained when, on feeding [^{15}N]ammonium chloride and α-ketoisovaleric or β-phenyl pyruvic acid to healthy and ureamic subjects on a low protein intake, nitrogen balance became less negative [326]. Further, the incorporation of ^{15}N into valine of plasma albumin was increased when α-ketoisovaleric acid was included in a valine-free diet. A similar increased incorporation of ^{15}N into both essential and non-essential amino acids occurred. Results obtained with β-phenylpyruvic acid were less conclusive.

An approximate quantitative assessment of the extent of urea nitrogen utilization may be obtained from the literature cited above. The absolute rate of urea nitrogen utilization for albumin synthesis has been determined by the use of a single combined injection of [^{15}N]urea, [^{14}C]urea and ^{125}I-labelled albumin in 10 ureamic patients and normal subjects [327]. Urea synthesis was determined from the dynamics of plasma [^{14}C]urea specific activity. Urea metabolism was then estimated from the relative rates of urea synthesis and urea appearance in urine and body water. Deconvolution analysis of plasma albumin ^{15}N enrichment and ^{125}I specific activity with time gave the cumulative incorporation of ^{15}N into total exchangeable albumin and permitted calculation of the absolute rate of urea nitrogen utilization for albumin synthesis. This rate was shown to be much higher in uraemic patients (mean 83.8 μmol h^{-1}) than in controls (mean 6.4 μmol h^{-1}) as was the efficiency of utilization of urea nitrogen for albumin synthesis, 1.3% and 0.2%, respectively. Taken in conjunction with earlier studies, however, these results suggest that restriction of dietary protein

in uraemia does not increase the reutilization of urea nitrogen for protein synthesis to a level of physiological significance. This protocol when applied to a patient with the gastrointestinal blind loop syndrome again demonstrated both increased urea hydrolysis and use of urea nitrogen for albumin synthesis compared to the normal subject [328].

Nitrogen metabolism in uraemic patients before and after dialysis has been investigated using [^{15}N]urea [329]. Results presented inferred that the effect of dialysis was to increase the incorporation of ^{15}N from labelled precursors into plasma and muscle protein despite the reduction of total non-protein nitrogen in body fluids, resulting from the dialysis. Alterations in the pattern of labelling of plasma and muscle protein as a result of dialysis resembled changes observed in uraemic patients fed low protein diets supplemented with essential amino acids and similarly, the negative nitrogen balance was reduced.

In another study this group of investigators have shown that urea nitrogen can be utilised for muscle protein synthesis in uraemic patients and that the administration of essential amino acids stimulated the utilization of nitrogen-containing metabolites for protein synthesis [330]. The ^{15}N content of amino acids from hydrolysates of plasma and muscle protein following the intravenous administration of [^{15}N]urea in ureamia, post-traumatic catabolism and in a normal subject were compared [331]. The distribution of label in amino acids supported the hypothesis that histidine is an amino acid which is essential in severe uraemia.

The fate of labelled ammonia and urea in children in the acute stage of marasmus and kwashiokor has been reported [332]. In the two forms of malnutrition, retention of both labels was high although ammonia nitrogen was retained to a greater extent than urea nitrogen. Additionally, it was shown that in marasmic children, oral [^{15}N]ammonia or intravenous injection of [^{15}N]urea resulted in both plasma proteins and erythrocytes becoming labelled. This did not occur in recovered children. In this study, highly labelled urea was used which when hydrolysed in the body would yield two $^{15}NH_3$-labelled molecules. The chances of recombination of NH_3-labelled molecules, within a vast pool to reconstitute doubly labelled urea are remote. Analysis of ^{15}N content of urea nitrogen can be effected by direct reaction with hypobromite. This is a monomolecular reaction [333] such that both atoms of a nitrogen molecule are derived from a common urea molecule. As a result, urea retains its isotopic identity and measuring the ratio of nitrogen molecules of masses 29 and 30 provides an estimate of the rate at which newly formed urea is produced from ammonia that was derived from the administered [^{15}N]urea. In the normal child, the *m/e* 30/29 ratio decreased exponentially, indicating that a constant proportion of body urea was being degraded and resynthesized. In a marasmic child however, the ratio was not exponential in decline and fell at a faster rate compared to the normal.

Quantitative studies of urea metabolism in malnourished and recovered children were designed to measure urea synthesis rate, the extent of urea recycling and its utilization for protein synthesis [334]. Estimates of these parameters were obtained on high and low dietary protein. [^{15}N]urea was administered by intragastric tube until a plateau enrichment was obtained for urinary urea N, and by inference in the urea precursor pool. Calculations were based on the level of urinary urea N at plateau. In 6 recovered children fed high protein, 68% of urea produced was excreted whilst of the remainder 50% was used for protein synthesis and 40% for resynthesis into urea. The remainder was accounted for as other metabolic processes. On reducing the protein intake, retention of synthesized urea was 70% of which only 9% was recycled as urea. The remainder was used for protein synthesis and other metabolic processes. Malnourished children showed similar trends in [^{15}N]urea handling though a greater amount of urea nitrogen was channelled into metabolic processes other than protein synthesis. Clearly these results demonstrate a far greater utilization of urea nitrogen for protein synthesis than reported in adults [327].

In isolated studies, ^{15}N labels have been employed to investigate inherited enzyme defects. Thus it has been reported that the excretion of ^{15}N label following the oral administration of ^{15}N-labelled ammonium chloride or urea, was elevated in an infant with congenital hyperammoniumaemia [335]. This condition results from a low hepatic carbamoyl phosphate synthetase activity. Despite the difference in isotope excretion pattern compared with matched controls, no special metabolic handling of the labelled ammonia was elucidated. A similar negative result was obtained in a male patient with argininosuccinic aciduria. The rates of synthesis and excretion of labelled urea, following an oral dose of [^{15}N]-ammonium lactate, were quantitatively similar to those obtained in a control subject [336]. This result was achieved despite the fact that the hepatic activity of arginosuccinase in the affected patient was less than 10% of normal. A later investigation in the same patient employed [^{14}C]citrulline and [^{15}N]ammonium lactate to monitor the appearance of both labels in argininosuccinic acid and urinary urea [337]. Whilst a precursor-product relationship was obtained between plasma citrulline and urinary argininosuccinic acid, no such relationship was found between argininosuccinic acid and urea. It was thus suggested that a large proportion of the urinary argininosuccinic acid was from extra-hepatic synthesis.

Creatine–creatinine

Early studies showed that when rats were fed [^{15}N]creatine it was deposited in the tissues and the extent of ^{15}N-labelling of urinary creatinine indicated that it originated from creatine [338]. On feeding [^{15}N]creatinine, rapid urinary excre-

tion followed, and no labelling of body creatine was observed, indicating that creatine–creatinine conversions were biologically irreversible. When a human subject was fed isotopic creatine for 38 days followed by [^{15}N]guanidoacetic acid for 10 days, on an otherwise creatine-free diet, the rate of endogenous creatine turnover was calculated to be 1.64% per day [339]. These investigators also showed, in a separate study, that the administration of methyltestosterone increased the rate of creatine synthesis in the body [340].

In a variety of clinical situations, it is important to have an estimate of muscle mass and to be able to monitor changes in this large protein pool. The most widely used method to obtain this estimate is based on a 24-h creatinine excretion [341]. The relationship found between daily creatinine output and muscle mass has been variously expressed by authors [342–344], though the validity of this procedure has been questioned [345].

The basis of this relationship is that urinary creatinine is the only degradation product of creatine phosphate [338] that cardiac and skeletal muscle contribute more than 90% of body creatine [346] and that creatine phosphate to creatinine degradation occurs at a fixed rate (non-enzymatically) [347]. Thus normally, the rates of formation and excretion of urinary creatinine would depend on the size and turnover rate of the creatine pool, and the relationship between muscle mass and creatine excretion would depend primarily on muscle creatine content.

Recently a study to investigate the validity of the concepts outlined above has been performed on 8 children who had recovered from protein calorie malnutrition [348]. Intravenous [^{15}N]creatine was used to label the creatine pool whose turnover rate was calculated from the single exponential decay curve of urinary [^{15}N]creatinine. Muscle mass was estimated from the creatine pool size and muscle creatine content obtained by analysis of percutaneous muscle biopsies. Results obtained from this study showed that creatine turnover was 2.1% per day (range 1.5–2.6) and that muscle creatine concentration was 2.5 μg per μg DNA (range 1.7–3.9). Muscle mass expressed as a percentage of body weight varied from 15 to 37 and the muscle mass equivalent to a daily excretion of 1 g of creatinine from 14 to 32 kg (mean 18.6 ± 6.6 kg). This mean value of 18.6 kg of muscle per gram of daily urinary creatinine agrees with indirectly derived estimates [342] and that obtained from whole body analysis [349]. The large standard deviation of the mean value (±35%) suggests that daily creatinine excretion has a limited value as a predictor of muscle mass, at least in children.

Applying this technique to malnourished children, it was demonstrated, not surprisingly, that muscle mass was severely reduced [350]. There was a greater reduction in muscle mass per kg body weight in kwashiorkor than marasmic infants even after the loss of oedema in the former group. It was also apparent that the creatinine excretion underestimated the degree of muscle mass deple-

tion in malnourished infants. A definitive study of this nature applied to normal and diseased adult subjects would be of immense value, especially in the study of various myopathies.

Changes in body creatine pool size on withdrawal of dietary creatine have been measured in 4 healthy young men [351]. This study lasted 56 days on a creatine free diet. [^{15}N]creatine was administered intravenously on days 4 and 46, and the urinary [^{15}N]creatinine content measured for 10 days. Extrapolation to zero time provided the ^{15}N concentration of creatine immediately after administration of the label and thus the creatine pool was determined by dilution. The mean creatine pool was reduced from 152 g to 130 g after 42 days. Urinary creatinine expressed as a percentage of the total creatine pool for the 8 determinations was 1.68 ± 0.05, and reported to be independant of the pool size.

Uric acid

Uric acid is the urinary end product formed from the catabolism of the purine ring of ribonucleotides that are either of dietary origin or result from *de novo* synthesis. Purine biosynthesis *de novo* commences with the reaction of phosphoribosylpyrophosphate (PRPP) and glutamine to yield phosphoribosyl-1-amine, which in effect, unites the final N9 atom of the purine ring with the carbohydrate moiety. The ensuing cascade of reactions results in the formation of inosinic acid which may be further converted to adenylic and guanylic acids or degraded to uric acid. This degradation includes conversion to xanthine in the liver, where the action of hepatic xanthine oxidase yields uric acid. Clinical disorders of purine metabolism are reflected in elevated plasma and urinary uric acid levels. Gout is the syndrome which results from the crystallisation of the sodium salt of uric acid due to body fluids being supersaturated with the salt. This is due to either excessive production or reduced excretion of uric acid or to a combination of both these processes. ^{15}N-labelled precursors have been used to investigate uric acid synthesis, pool size and turnover in normal and gouty subjects.

Following the administration of [^{15}N]glycine to a normal subject, it was found that all four nitrogen atoms of uric acid carried excess ^{15}N [352]. Moreover, the degree of labelling was shown to decrease from N7 through N1, N9 and N3. It was also demonstrated that N7 and C4, C5 of the purine ring were derived from the intact glycine molecule [353], N1 from aspartic acid and N9 together with N3 from the amide nitrogen of glutamine. Thus the intramolecular variation of ^{15}N content stemmed from the degree of isotopic labelling exhibited by the constituent precursors.

Initial investigations using ^{15}N-labelled uric acid provided information with

regard to the miscible pool size and turnover rate [354–357]. Whilst these studies demonstrated that gouty patients had an increased miscible pool compared to normal, they provided no insight into the underlying metabolic defect. In normal subjects approximately one-third of all uric acid produced is broken down in the body. This is primarily by uricolysis and the remainder of the uric acid appears in the urine [354,358].

It has been calculated that some 200 mg of uric acid is excreted into the digestive tract daily and thus open to degradation by intestinal micro-organisms [359]. [^{15}N]Glycine was fed to two normal and one gouty subject and analysis of urinary nitrogenous end-products showed similar total nitrogen, urea and ammonia excretion [360]. However the labelled uric acid from the gouty subject displayed a higher maximum, a more rapid decline, and a threefold incorporation compared to the normals. These findings were confirmed in a later study designed to investigate the suppression of [^{15}N]glycine incorporation into urinary uric acid by [^{13}C]adenine in normal and gouty subjects [361]. The ^{13}C label enabled an estimate of the conversion of exogenous adenine to urinary uric acid. Adenine was found to inhibit the *de novo* synthesis of purines in both normal and gouty subjects demonstrated by the inhibition of the incorporation of [^{15}N]glycine into urinary uric acid. This suppression of incorporation was comparable to that produced by 4-amino-5-imidazolecarboxamide (AIC) [362].

The early labelled peak of ^{15}N incorporation [360] indicative of excessive uric acid production in gouty subjects was abolished by adenine or AIC administration.

Other reports have indicated that not only was the [^{15}N]glycine incorporation into uric acid elevated in subjects with primary gout but that the intramolecular distribution of label was altered compared with the normal [363]. Specifically, the initial ^{15}N(9 + 3 + 1) : total uric acid ratio was elevated in gouty subjects which led to speculation of an abnormality in glutamine or aspartic acid metabolism [364]. In a later report, selective degradations of uric acid showed ^{15}N(9 + 3) was raised thus implicating glutamine metabolism [365]. In addition these investigators found a reduced urinary [^{15}N]ammonium enrichment due to a defect in the production of ammonia from glutamine in the kidney. Later, more detailed studies suggested that the increased uric acid ^{15}N(9 + 3) of primary gout was a kinetic phenomena arising from the differential time course of precursor pool ^{15}N-labelling with respect to glycine and glutamine [366].

Haematological investigations

It was first demonstrated in 1945 that glycine was utilized for the synthesis of protoporphyrin in man [367], and it was possible with [^{15}N]glycine to show

that the life span of the labelled cohort of erythrocytes in normal man was 120 days [368]. In various anaemias, mean erythrocyte life may be appreciably reduced. There are reports of 85 days in pernicious anaemia and 42 days in sickle cell anaemia [369]. In two newborn infants, the average erythrocyte life span was calculated to be 85 and 91 days [370], whilst in children aged 4–14 months recovering from protein–calorie malnutrition, a normal red cell survival was demonstrated [371].

Subsequently it was shown using [^{15}N]glycine that all 4 nitrogen atoms in protoporphyrin were derived from that amino acid [372]. It is now known that one molecule of glycine and one molecule of succinyl-CoA condense to form one molecule of α-aminolaevulinic acid (ALA). Two molecules of ALA are then linked by the action of cytoplasmic ALA dehydrase to form porphobilinogen (PBG). Four molecules of PBG form a tetrapyrrole, uroporphyrinogen, and subsequently by decarboxylation and oxidation of the side chains, protoporphyrin IX is formed which combines with iron to form haem. Use has been made of both ^{15}N and ^{14}C precursors to elucidate the intermediate steps involved in haem synthesis [373–378]. Much of the early work covering the biosynthesis of porphyrin has been reviewed in detail [379].

Haem catabolism occurs in both the liver and gut. Microsomal haem oxygenase of the reticulo-endothelial cells and hepatic parenchymal cells convert haem to biliverdin, and thence rapidly to bilirubin via a reductase. In the gut reduction of methene and vinyl groups of bilirubin results in the formation of colourless urobilinogens which on oxidation produce orange stercobilins that appear in the faeces. All these pigments contain the 4 nitrogen atoms which were initially derived from glycine via haem.

In 1950 it was first shown that bilirubin arises from sources other than the destruction of circulating red cells [380]. Following the administration of [^{15}N]glycine to 2 normal subjects and its incorporation into protoporphyrin, red cells containing labelled haemoglobin haem appeared in the circulation where they remained for approximately 120 days. Their destruction was associated with a peak of labelled stercobilin excretion between approximately 100 and 140 days (the late peak). A peak of labelled stercobilin excretion was also found during the first few days, at the time when labelled haem was appearing in the circulation, subsequently termed the early labelled peak (ELP). By comparing the concentration of ^{15}N in haem and in stercobilin over the first 8 days, it was calculated that at least 11% of all stercobilin was derived from sources other than the destruction of mature erythrocytes. Soon after, a similar experiment was repeated in a normal subject and the ELP was shown to constitute at least 20% of total pigment production [381].

It was considered that the most likely source of the ELP was the destruction of erythroid cells within the marrow or shortly after reaching the circulation, i.e.

ineffective erythropoiesis. Support for the erythropoietic origin of the ELP came from a study in a patient with pernicious anaemia in which the ELP accounted for at least 40% of the total pigment produced [369]. Further evidence was the finding of an increase in the ELP with erythroid hyperplasia and a reduction in aplasia. [^{14}C]Glycine has been employed to demonstrate that the ELP was increased in man when hyperplasia was induced by haemorrhage [382]. Animal work has confirmed this observation and in addition it was found that the late peak was also increased, so that the ELP accounted for the same proportion of total pigment production as in control animals [383]. Similar findings have been reported in paroxysmal nocturnal haemoglobinuria [384] and in sickle cell anaemia [385].

In conditions where there is dyserythropoiesis, with or without hyperplasia, the ELP is greatly increased as has been demonstrated using either ^{15}N- or ^{14}C-labelled glycine. Examples include pernicous anaemia [386,387], thalassaemia [387,388], 'shunt' hyperbilirubinaemia [389], sideroblastic anaemia [387,390] and anaemia of rheumatoid arthritis [391].

The labelling of plasma bilirubin has been studied following the administration of [^{14}C]glycine and [^{14}C]ALA to normal subjects [392]. After glycine there were two peaks of labelling, one at 12–24 h and the second at 3–4 days, the period of maximal incorporation of ^{14}C into haemoglobin haem. After ALA, only the initial peak was seen, and there was minimal activity in haemoglobin haem. From this investigation it was concluded that the initial peak seen after both glycine and ALA was of non-erythropoietic origin and that it resulted from hepatic haem breakdown.

In order to calculate the total amount of early labelled bilirubin produced after giving a labelled precursor of haem synthesis, both the isotope concentration of the bilirubin (in the case of ^{15}N, this is the same as that of faecal stercobilin) and the total amount of bilirubin produced per day must be measured. The percentage incorporation of the precursor can then be calculated. When glycine is the precursor, comparison of the percentage incorporation into the ELP with the percentage incorporation into haem (equivalent to the late peak) allows the ELP to be expressed as a fraction of total bilirubin production, and hence in absolute values.

Recently this type of study has been undertaken in order to quantitate ineffective erythropoiesis in normal subjects [393] and in patients with a variety of haematological disorders [387]. In these investigations the incorporation of [^{15}N]glycine into early labelled bilirubin and haemoglobin haem was measured. [^{14}C]Bilirubin clearance was used to estimate total bilirubin production rate [394], and hepatic haem turnover was calculated from the incorporation of α-amino [^{15}N]laevulinic acid into early labelled bilirubin. The relative contribution of ineffective erythropoiesis in the production of anaemia found in these

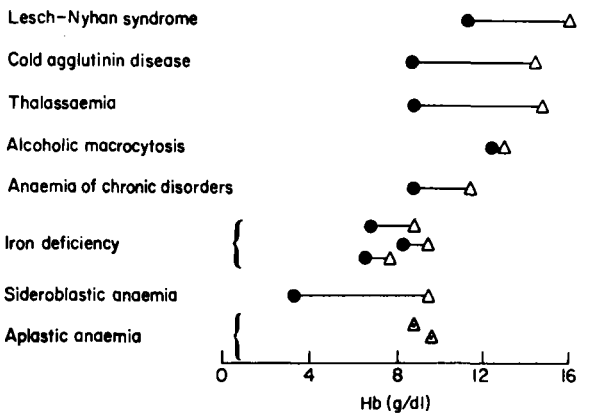

Figure 1.8. The role of ineffective erythropoiesis in the production of various anaemias. The actual haemoglobin (Hb), •, is compared to the potential Hb, △, which would be achieved if ineffective erythropoiesis were within the normal range

studies is depicted in *Figure 8.8* which compares actual and potential haemoglobin levels. Thus in patients with Lesch–Nyhan syndrome and megaloblastic anaemia, cold agglutinin disease or thalassaemia, ineffective erthropoiesis contributed totally to the anaemia presented. In aplastic anaemia and iron deficiency anaemia ineffective erythropoiesis did not contribute to the anaemia.

Genetically transmitted porphyrias that are classified as either erythropoietic or hepatic, according to the apparent site of primary defect in porphyrin metabolism, have been studied using [^{15}N]glycine and α-amino [^{15}N]laevulenic acid. Thus in congenital erythropoietic porphyria (CEP) there was an increased incorporation of glycine into early labelled bile pigment [381] which was explained as an increased turnover of bone marrow haems. In erythropoietic protophorphyria (EPP), the synthesis of erythropoietic haem was demonstrated to be normal [395] whilst in separate studies an enhanced catabolism of hepatic haem has been suggested as a contributory factor to the early labelled peak observed [396,397].

Protein metabolism

Nitrogen balance studies, when performed under strict supervision, can yield information regarding the overall state of nitrogen metabolism in the body. However, a change in nitrogen balance may be a reflection of a change in the rate of protein synthesis or protein degradation, or both [398]. The use of isotopic labels in conjunction with balance studies have provided quantitative infor-

mation with regard to separate components of protein metabolism in the human subject. This information has clinical significance in the treatment of obesity, stress, post-operative trauma and the choice of long-term parenteral nutrient.

The first measurements of protein turnover in a human subject were performed in 1949. Since that time several models have been proposed to give true estimates of protein turnover, or at least permit intra-subject comparison of synthesis and catabolism. The great majority of estimates of protein turnover in the human subject have involved the use of [^{15}N]glycine administered either as a pulse label or by constant infusion, and measurement of the resultant ^{15}N label in the urine (^{15}N total, [^{15}N]urea or [^{15}N]ammonia). Detailed treatment of the underlying theory of these models has been presented in 2 excellent reviews [399,400]. Initial studies used [^{15}N]glycine, administered as a single dose (pulse labelling) and the rate of protein turnover was calculated from the curve of ^{15}N excretion over 48–60 h [401]. The excretion curve obtained was fitted to the equation:

$$\frac{\lambda_E}{\lambda_0} = A(1 - e^{-Bt})$$

in which A and B were constants, λ_E represented the amount of isotope excreted in time t and λ_0 was the dose of ^{15}N. It was assumed that the label would mix freely with the metabolic nitrogen pool and either be involved in protein synthesis or excreted as urea. Re-entry of ^{15}N label from the catabolism of newly synthesized protein and faecal loss of ^{15}N were not included in the model. Theoretically when all the label had cleared the metabolic pool, the level of label excreted in the urine should plateau, corresponding to the constant A. In fact plateau excretion of label was not achieved during the experiment and A was calculated from the initial excretion curve taken as a single exponential. Results obtained on five normal subjects gave a mean value for protein synthesis of 1.36 g per kg body wt per day. Despite the limitations of the model it was subsequently used by several groups of workers. Protein synthesis was shown to be approximately twice as fast in children when compared with adults using ^{15}N-labelled aspartic acid [402], and was more rapid in young adults than in elderly subjects [403]. In animals, growth hormone administration was shown to promote the rate of protein synthesis [404]. Other workers modified the above method and used glycine [405] aspartic acid [406] or phenylanine [407] in their studies. Protein turnover was calculated from the cumulative ^{15}N excretion curve at infinite time as they had demonstrated the inadequacies of using the early excretion curve. It was found that the 3 amino acids used gave similar results.

A modification of the original model was the addition of a urea pool [408], the $t_{1/2}$ of which is some 8–10 h. Involvement of the urea pool would lengthen

the time required to achieve plateau labelling in the urine (A) but not affect the ultimate level of excreted ^{15}N. In this case protein synthesis was calculated from the maximum ^{15}N label which in practice was 3–4 h, obtained in urinary urea following a single dose of glycine. Precise measurement of this peak urea labelling proved difficult and results obtained in normal subjects showed large variations. Consistent results were obtained however when repeated estimates were performed on the same subject under comparable dietary conditions, though not when protein or calorie intake were varied [409]. Pathological conditions investigated using this model include the refeeding of a patient with anorexia nervosa in which calorie and nitrogen intakes were varied independently [410]. Protein synthesis was relatively constant throughout the study whereas the size of the metabolic pool and its turnover rate varied with changes in calorie intake. The administration of triiodothyronine to patients with primary myxoedema was shown to restore their initially depressed levels of protein synthesis to normal [411]. The administration of this hormone to a control subject decreased protein synthesis and it was suggested that an excess of the hormone could have resulted in the altered rate of protein synthesis observed.

Protein metabolism was estimated in 3 premature infants between 40 and 70 days after birth [412]. The rate of protein synthesis and the metabolic pool size were greatly increased when compared with normal adult values.

Within a year, another model was presented that required the measurement of ^{15}N excretion in the urine for 15 days following a single dose of [^{15}N]glycine [413]. This model contained the metabolic and urea pools of previous systems and in addition a rapidly turning over protein pool (viscera) and a slow pool (muscle). Compartmental analysis of the excretion curve gave 3 exponential slopes and intercepts from which the various pool sizes and inter-pool nitrogen transfer could be calculated. Whilst this study was probably the first instance of strict dietary control during the investigation of protein synthesis, the time scale involved precluded its use becoming more than academic. However, these same investigators demonstrated that in man immobilization by means of a plaster cast greatly reduced protein synthesis [414].

Theoretically any excreted end product may be used to calculate the rate of protein synthesis provided it is derived from the homogeneous metabolic pool which also serves as the precursor pool for protein synthesis [415]. The limitations of sampling in the human subject have led investigators to use urea, or more recently ammonia, as the end product for isotopic measurements. Clearly, neither metabolite complies strictly with the above requirement, as urinary urea is of hepatic origin only whilst protein is synthesized in all tissues. The advantage of using this end product is that with a large body urea pool, turning over relatively slowly, there is a delay in excretion of label following a single ^{15}N dose. Similarly ammonia is not formed throughout the body but mainly in

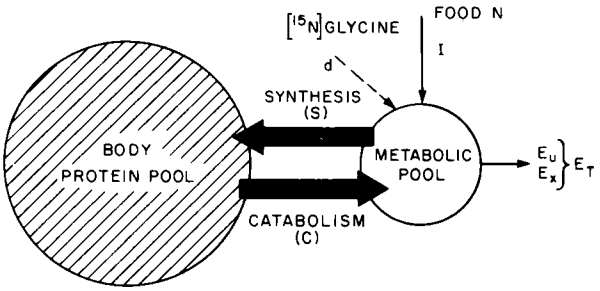

Figure 1.9. Model used to study dynamic aspects of whole body nitrogen–protein metabolism [420]. I, S and C are the dietary intake, protein synthesis and catabolism respectively; E_T, E_U and E_X are total, urinary urea and non-urea nitrogen excreted, respectively. All values are expressed as mgN Kg^{-1} day^{-1}

the kidney [416,417] from glutamine, which originates mainly from muscle. In spite of these apparent objections, ammonia has been employed as the end product for isotopic measurements to estimate protein turnover. Obese patients fed a normal diet initially followed by 3 wk on a low calorie diet containing 50 g protein had similar rates of protein synthesis. A low calorie diet containing no protein reduced the rate of protein synthesis by 50% in 3 wk [418,419]. The results obtained with ammonia were lower than corresponding urea estimates of protein synthesis, but the 'rank order' was similar. The authors do not claim that the results obtained using ammonia are necessarily correct, but stress the potential use of this technique for rapid clinical comparisons.

During the decade 1959–1969 few attempts were made to measure protein turnover in human subjects. In 1969 the first report of a constant infusion technique, applied to malnourished and recovered infants, was reported [420]. It is worthwhile considering briefly the model and its fundamental assumptions as, with slight modification, it has been employed almost exclusively in measurements of protein metabolism published to date. The method involved the intravenous or intragastric infusion of [^{15}N]glycine for some 30 h at which time the urinary urea had attained a plateau value with respect to ^{15}N enrichment. The simultaneous measurement of nitrogen balance permitted calculation of protein synthetic and catabolic rates. *Figure 1.9* depicts the model used in this type of study. The model reverted to a single protein pool, which is undoubtedly an oversimplification, and did not provide an estimate of the size of the metabolic pool. The assumptions required were, that ^{15}N and ^{14}N were treated in an identical manner, the metabolic pool size remained constant, dietary amino acids and those resulting from protein catabolism were similarly treated and that there was minimal re-entry of isotope into the metabolic pool during the plateau. Implicitly [^{15}N]glycine was assumed to be representative of total amino nitrogen. The

overall equation for this model when steady state conditions prevailed, showed that the rate (Q) at which amino N enters the metabolic pool (P) equals the rate at which it leaves the pool. Thus:

$$Q = I + C = S + E_T$$

Flux = Intake + Catabolism = Synthesis + Total excreted N

where Q was the total turnover rate (or flux) of amino N through the metabolic pool. Thence the percentage, F, of the infused isotope that was excreted as urea was the same as the percentage of total amino N excreted as urea from the metabolic pool.

$$F = \frac{e_u}{d} = \frac{E_u}{Q} \text{ where } e_u \text{ is rate of excretion of } {}^{15}N \text{ as urea}$$

$$\therefore Q = \frac{E_u}{F}$$

Rather surprisingly the results from this investigation showed that malnourished infants were turning over body proteins at almost double the rate of recovered infants. These findings seemed to conflict with animal studies where it was found that dietary restriction resulted in a rapid fall in muscle protein synthetic rate [421]. This apparent anomaly was later explained by the fact that at the time of the study the malnourished children were on a recovery diet. Recently, malnourished children have been studied on a maintenance diet, a recovery diet, and when recovered [422]. The rates of protein turnover, synthesis and catabolism during the rapid growth phase on a recovery diet were shown to be higher than in either the malnourished or recovered state. The only modification to the original protocol was that the [^{15}N]glycine was administered intermittently, at two hourly intervals with feeds.

Other investigators following this protocol have measured protein synthetic and catabolic rates in patients undergoing elective orthopaedic surgery [423]. There was a failure to demonstrate any significant alteration in either rate following surgery despite the fact that the patients were in negative nitrogen balance. A more recent publication using constantly infused [^{15}N]glycine [424] and studies with [^{14}C]leucine have indicated that a reduction in synthesis rather than increased catabolism gives rise to post operative nitrogen losses [425]. There is now a growing bank of results showing changes in the rate of protein synthesis, catabolism and whole body flux at various ages in the human subject. The constant infusion technique has been used for estimates of body nitrogen metabolism in neonates [426] and newborn [427], young adults at two levels of protein intake [398,428] and elderly subjects [429]. It has also been used to estimate the rate of protein synthesis in children after thermal injury [430].

Table 1.4. MEASURED RATES OF PROTEIN SYNTHESIS IN HUMAN SUBJECTS AT VARIOUS AGES USING CONSTANT INFUSION OR SHORT INTERVAL FEEDING OF ^{15}N GLYCINE AND URINARY UREA AS END PRODUCT

Age group	*Age (range)*	*Body weight (kg)*	*No. of studies*	*Total body protein synthesis ($g\ kg^{-1}\ d^{-1}$)*	*Reference*
Neonates	1–45 d	–	5	26.3 ± 7.0	426
Newborn (prem.)	1–46 d	1.94 ± 0.6	10	17.4 ± 7.9	427
Infant	10–20 m	9.0 ± 0.5	4	6.9 ± 1.1	420
Young adult	20–23 y	71 ± 15	4	3.1 ± 0.2	398
Adult [a]	31–46 y	69 ± 8	5	3.5 ± 0.5	431
Elderly	69–91 y	56 ± 10	4	1.9 ± 0.2	429
Elderly	66–91 y	61 ± 10	6	3.5 ± 0.2	435

[a] Constant intravenous infusion of L-[α-^{15}N]lysine.

Table 1.4 shows the values obtained recently for the different parameters of nitrogen metabolism with urea as end product [420].

In an attempt to exclude the use of urea from protein turnover measurements, [^{15}N]lysine has been infused intravenously until the free lysine in the plasma achieved plateau labelling [431]. From the lysine flux, and its mean representation in all body proteins, turnover rates were calculated in a manner analogous to that used in radioactive amino acid studies [432]. In addition, serial muscle biopsies taken 14–16 h apart when plasma [^{15}N]lysine plateau conditions prevailed, enabled muscle protein turnover rate to be calculated. This was found to account for 53% of whole body protein turnover.

Of the various methods for estimating protein synthesis that have been outlined in this section, none can claim to be ideal. Valid objections have been raised when using a single injection of an ^{15}N label [407,409], not the least of which is the inevitable rapidly changing ^{15}N enrichment of the precursor pool from which protein synthesis occurs. The choice of amino acid should be such that the proportion of the label excreted in urinary urea is equal to the proportion of total amino nitrogen excreted in urinary urea [420]. This applies equally to the use of ammonia. Presumably low cost and availability have dictated that glycine be used more than any other amino acid in protein metabolism studies in spite of the fact that it does not readily undergo transamination and evidence has been presented for the existence of more than one glycine pool [433]. Comparative studies, however, have obtained reasonable agreement when using glycine and aspartic acid [409,434], ^{15}N-labelled egg protein [420] and [^{14}C] leucine [435] in the same subjects. This latter finding is encouraging in that the

calculation of results for each tracer rely on an unrelated set of assumptions. Several amino acids have been shown to be unlikely to provide sensible estimates of whole body protein synthesis [436,437].

Finally, both constant infusion and pulse label techniques using isotopic labels to estimate whole body protein turnover share the common premise that there is a homogeneous metabolic nitrogen pool of which the plasma constitutes an integral part. That this is in fact an oversimplification, has been shown from animal studies [438]. Despite these, and other objections, work will continue in the search for a reliable method for the estimation of protein synthesis, catabolism and turnover in man. To the clinician such a method would provide information about nitrogen loss from the body resulting from malnutrition, post-operative trauma, burns or severe infection and perhaps more importantly an indication of the success or otherwise of the specific therapeutic regime implemented.

Miscellaneous

A recent study was undertaken to compare the efficacy of human growth hormone (HGH) and the androgen, oxandrolone, in the longterm treatment of growth deficient children [439]. Nine day nitrogen balance studies were performed at intervals during treatment with HGH or oxandrolone and compared with concurrent ^{15}N retention measurements following the oral administration of [^{15}N]glycine. Both methods demonstrated an increased nitrogen retention during therapy and the advantages of the isotopic method were presented. A similar comparison of nitrogen balance and isotope retention measurements has been reported in animals [440]. In that instance, the anabolic and catabolic effects of growth hormone and corticotropin respectively, were demonstrated. ^{15}N-isotope balance studies have also proved capable of detecting affected males, with testicular feminization, and healthy female carriers [441].

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2 Membrane-Active Antimicrobial Agents

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INTRODUCTION

The first demonstration of a possible connection between membrane damage and bactericidal activity was made by Hotchkiss [1] who studied the action of the polypeptide antibiotic tyrocidin upon staphylococci. Treatment of the cells with tyrocidin caused a rapid release of phosphorus- and nitrogen-containing material. The amount released was similar to that obtained by prolonged extraction of the cells with 5% trichloroacetic acid at 0°C, a procedure which releases the low molecular weight components of the metabolic pool. It was therefore concluded that tyrocidin damaged the permeability barrier of the cell membrane. Similar investigations by Gale and Taylor [2] confirmed these findings by showing that tyrocidin released from the cells certain free amino acids which are normally maintained at high intracellular concentration. In this case, a quantitative relationship between leakage, tyrocidin concentration, and loss of cellular viability was established. Some other unrelated bactericidal compounds including cetyltrimethylammonium bromide (CTAB) and phenol gave similar results.

A more convenient method of detecting the release of cytoplasmic constituents following damage to the cell membrane was developed by Salton [3]. He measured the leakage of material absorbing light in the 260 nm wavelength region and found a linear relationship between bactericidal activity and leakage from cells of *Staphylococcus aureus* and *Escherichia coli* exposed to CTAB over a given concentration range. The 260 nm-absorbing material was made up of purine- and pyrimidine-containing compounds, i.e. nucleosides and nucleotides presumed to be associated with the metabolic pool of the organisms. Detection of the release of 260 nm-absorbing material from treated cells is now generally regarded as being symptomatic of cell membrane damage and is routinely used in studies on the mechanism of action of antimicrobial agents [4,5]. In this way, many agents of diverse chemical composition have been shown to damage the cell membrane. Phenols, alkylphenols, chlorinated phenols and cresols, chlorhexidine, quaternary ammonium compounds and polymyxins all elicit leakage and consequently they can be conveniently classed together as membrane-active antimicrobial agents [6,7]. At the relatively high concentrations at which they are usually employed as bactericides, these agents produce a general disorganization of the cell membrane, probably by denaturing protein molecules. However, with some of the agents, more subtle effects are apparent when lower concentrations are studied. For example, chlorhexidine inhibits cation transport and the membrane-bound ATPase at concentrations below that required to produce leakage [8].

The bacterial cell membrane is the site of many important cellular functions: transport mechanisms for ions and metabolites, biosynthesis and assembly of

wall components, and energy-generating systems involving electron transport and oxidative phosphorylation. Among the antibiotics and antimicrobial agents which have a selective action upon such functions are the ionophores. These compounds interfere with transport mechanisms by acting as artificial cation carriers in the cell membrane. They destroy ion gradients that are generated across the membrane by the cells in order to promote uptake of metabolites. This is a field of intensive investigation in which the ionophores are playing an important part as biochemical tools.

Although the main part of this review is concerned with agents which damage the cytoplasmic membrane, a section is included on the outer membrane of Gram-negative bacteria. The outer membrane is thought to be responsible for the resistance of Gram-negative organisms to many antibiotics, dyes and detergents which are active against Gram-positive cells [9]. The basis of the resistance is the penetration barrier presented by the other membrane. Chelating agents much as ethylenediaminetetracetic acid (EDTA) used in conjunction with an organic base such as tris (hydroxymethyl) aminomethane (Tris) damage the outer membrane and render it permeable to antibiotics which are normally excluded. This is clearly of potential medical importance and an understanding of outer membrane permeability is necessary in order to design agents which are effective against Gram-negative organisms.

THE CYTOPLASMIC MEMBRANE

STRUCTURE AND COMPOSITION

When viewed in thin section under the electron microscope, the cytoplasmic membrane of bacterial cells has the same double-tracked profile as the 'unit' membrane found in higher cells [10]. Sectioning reveals little detail of membrane structure apart from the thickness (70–80 Å) which is similar to that of membranes from other sources. The major components of bacterial membranes are protein (50–70%) and phospholipid (20–30%), the proportions varying from organism to organism [11].

Of the many models describing the organization of phospholipid and protein in the cell membrane [12], the 'fluid mosaic' model [13] is currently thought to be the most realistic. The protein molecules are distributed throughout a fluid phospholipid bilayer, some on either side of the membrane and some protruding through and exposed on both sides. Hydrophilic side groups of the polypeptides, i.e. COOH, OH and NH_2 groups, are exposed on the surface of the bilayer adjacent to the polar portions of the phospholipid molecules. In this configuration the groups are hydrated and can interact with metal ions. Hydrophobic portions

are embedded in the hydrophobic core of the bilayer where they can associate with the hydrocarbon chains of the phospholipids. Part of the polypeptide chains of the proteins exist in the ordered α-helical configuration. Thus several types of molecular interaction: ionic attraction, hydrogen honding, London–van der Waals forces and 'hydrophobic bonding' contribute to the stability of the fluid mosaic membrane and play a part in holding it together. The forces operate between adjacent lipid molecules, between lipids and proteins, and also between protein molecules [14]. Covalent bonding probably does not occur since membranes can be readily dissociated with organic solvents and detergents and disrupted by ultrasonic irradiation [15]. The relative contribution of each type of molecular interaction to the stability of the membrane is unknown; it might vary between different organisms or even from one region of the membrane to another. However, it is clear that the intrusion of molecules that interfere with the balance of the molecular interactions will affect the integrity and function of the membrane or even lead to its disruption. This is the molecular basis of action of many of the non-specific membrane-active antimicrobial agents.

FUNCTION

The cytoplasmic membrane is the site of many important cellular functions: active transport, electron transport and oxidative phosphorylation, secretion of exocellular protein toxins and enzymes, biosynthesis of cell wall components (e.g. peptidoglycan, lipopolysaccharide, teichoic acid), protein and phospholipid biosynthesis, and chromosome anchoring [16]. The enzymes and other specialized molecules which carry out these functions, i.e. dehydrogenases, cytochromes, ATPase, wall polymer synthases and their associated lipid intermediates are all located within the cell membrane [16]. They are probably not distributed randomly throughout the membrane but are organised in discrete regions of 'domains' each designed to carry out a particular function. Processes such as wall polymer biosynthesis or electron transport require the participation of many macromolecules in a coordinated manner and this could only occur efficiently if the molecules are in close proximity. It is thought that the phospholipid and protein components of the cell membrane play an active part in providing the specialized microenvironments necessary for each of the functions. For example, the proportion of different lipids present in the membrane, the nature of their polar head groups, the chain length and degree of unsaturation of their fatty acid residues are factors which control the fluidity of the bilayer [17]. It has been suggested that the presence of different phospholipids with a range of fatty acid residues would permit the simultaneous existance of fluid and solid 'domains' in the membrane [18] thereby concentrating selected metabolites in local regions, segregating dissimilar processes, and bringing together related ones

[19]. Evidence for the participation of membrane components in this way comes from the selective lipid requirements displayed by certain membrane enzymes and the release of enzyme-lipid complexes from membranes with detergents [20–23]. Visual evidence for the existance of specialized regions of the cell membrane is provided by the appearance of negatively-stained preparations under the electron microscope. Thus in *Micrococcus lysodeikticus* ATPase activity is associated with a membrane-bound complex made up from 6 subunits surrounding a central particle [16].

Many approaches have been used to study membrane structure: electron microscopy of freeze-fractured and freeze-etched preparations, localization of antigens using ferritin-labelled antibodies, crossed immunoelectrophoresis, chemical probes and cross-linking agents, and instrumental techniques such as nuclear magnetic resonance and electron spin resonance spectroscopy. Their contribution to our present understanding of membrane anatomy has been reviewed by Salton and Owen [24].

METHODS OF DETECTING MEMBRANE DAMAGE

The classical method of detecting membrane damage is to measure the release of low molecular weight cell components into the medium following exposure to the antimicrobial agent [4,5]. Purine- and pyrimidine-containing compounds are conveniently determined by their absorbance at 260 nm [3]; amino acids, sugars, NH_4^+ and phosphate by chemical methods [25]; and metal ions such as K^+, Na^+ or Mg^{2+} by flame photometry or atomic absorption spectroscopy [25]. In some cases greater sensitivity can be achieved by preloading the cells with a radioactively-labelled component, e.g. [^{14}C]alanine and [^{14}C]glutamic acid [26], [^{14}C]glucose and [^{32}P]phosphate [27], $^{42}K^+$ [28] and $^{86}Rb^+$ [29], and measuring the leakage by liquid scintillation counting.

All of these methods require prior removal of the cells from suspension and, even when rapid membrane filtration is employed [30], some uncertainty in the leakage time course is introduced. Interference with the assay of material released from the cells by components of the buffer system or by the drug itself is another problem which can sometimes be obviated by careful choice of buffers [31] and extraction of the drug into an organic solvent [32]. Measurement of leakage from growing cells is generally precluded by the composition of the medium, components of which interfere with the assays. However it might be possible to detect release of 260 nm-absorbing material from cells grown in minimal medium of low UV absorbance [33].

By immersing a K^+-sensitive ion-selective electrode in the cell suspension the leakage of K^+ induced by addition of a drug can be measured *in situ* [34]. Using

this technique accurate measurements can be made of the rapid efflux of K^+ which is thought to be one of the first indications of membrane damage [31,34–36]. Rate of loss of K^+ from cells or the time required to reach a standard rate of leakage of K^+ have been used to estimate the extent of membrane damage and the sensitivity of cells to membrane-active agents [37,38].

Membrane damage can also render cells permeable to dyes and enzyme substrates that are normally excluded. Penetration of dyes which fluoresce when bound to proteins [39] or to nucleic acids [40] following drug treatment has been correlated with membrane damage. Similarly, stimulation of the reduction of tetrazolium dyes by dehydrogenases results from increased permeability of the substrate [41]. Methods used to measure release of metal ions [29] or labelled amino acids [26] can also be used to detect stimulation of uptake. Changes in the rate of respiration of cell suspensions induced by drug treatment can be accurately monitored with an oxygen electrode [42]. The sensitivity and rapid response of this technique enables the results to be compared directly with K^+ leakage measured with an ion-selective electrode [31,43].

Measurement of the effect of agents upon osmotically-stabilized suspensions of protoplasts and spheroplasts provides another sensitive means of detecting membrane damage. At low concentrations many membrane-active agents produce rapid lysis which can be detected by a fall in turbidity [32,44]; at higher concentrations some agents cause an increase in turbidity which is due to coagulation of the cytoplasm [31]. By carrying out similar experiments in osmotically-stabilized suspensions containing various salts, it is possible to detect changes in membrane permeability towards particular ions [45]. Thus studies on various skin germicides have shown that tetrachlorosalicylanilide (TCS) increases membrane permeability towards NO_3^- [45] whereas CTAB and chlorhexidine produce general membrane damage and a non-selective increase in permeability [45,46].

The results of gross membrane damage are sometimes visible under the electron microscope. The effects usually observed are the loss of cytoplasmic material following enzymic degradation of polymers and extensive leakage of the products [47–50]. Breaks in the membrane have been observed in a number of organisms following exposure to phenethyl alcohol [51]. More subtle effects upon the cell membrane, such as inhibition of membrane-bound enzymes or transport systems do not give rise to noticeable ultrastructural changes.

According to Mitchell's chemiosmotic theory [52,53] active transport, oxidative phosphorylation and ATP synthesis are driven by a 'protonmotive force' which is made up of two components: an electrical potential gradient and a pH gradient across the cell membrane. Agents which conduct H^+ ions across the membrane destroy the pH gradient and thereby reduce the protonmotive force. It is possible to measure the pH gradient and to monitor its attenuation by

uncoupling agents using a glass electrode [46,54,55] or a metabolically-inert indicator [56]. The electrical potential across the membrane can be measured using dibenzyldimethylammonium cation (DDA^+) [57]. The distribution of DDA^+ across the cell membrane at equilibrium is determined by the membrane potential; measurement of the concentration of DDA^+ inside and outside of the cell enables the potential to be calculated from the Nernst equation [58]. The construction of an electrode which selectively measures DDA^+ concentration [59] should facilitate measurements of membrane potential.

Much useful information on the mechanism of action of membrane-active agents has come from studies of their effects upon artificial lipid membranes [60]. Bilayer films less than 100 Å thick can be prepared from a variety of lipids [61–63]. Using suitable techniques [61–63] the effect of membrane-active agents upon a number of physical and electrical properties of the bilayers can be investigated. Among the properties measured are surface pressure [64,65], electrical resistance [66] and the permeability of ions [67,68], water and non-electrolytes [69]. By varying the composition of the bilayer films it is possible to investigate the influence of the nature of the lipid molecules upon the sensitivity of the films to membrane active agents [70]. Liposomes are closed concentric vesicles prepared by careful dispersion of artificial lipid bilayers [71]. Marker molecules such as glucose can be trapped inside the liposomes [72]; the release of glucose following treatment with membrane-active agents can then be used to detect damage to the integrity of the liposome membrane [65].

AGENTS WHICH DISRUPT THE CYTOPLASMIC MEMBRANE

ORGANIC SOLVENTS

Organic solvents such as chloroform and toluene have been used for many years to prevent microbial growth in aqueous solutions. Many solvents, e.g. toluene [73], phenethyl alcohol [28], ethanol [74], and butanol [75] elicit a rapid release of cytoplasmic constituents from microbial cells. Disorganization of the membrane is probably due to penetration of the solvents into the hydrocarbon interior of the membrane. The amount of damage caused to the membrane seems to depend upon the concentrations of solvent in the lipid phase: equal degrees of lysis of protoplasts of *M. lysodeikticus* are produced by equal thermodynamic activities of aliphatic alcohols [76].

Addition of toluene to exponentially-growing cells of *E. coli* causes massive loss of material: up to 85% of ribosomal RNA and 25% of cellular protein, the precise amount depending upon the level of toluene employed [73]. The cells

are not lysed by toluene [73] but partial dissolution of the membrane has been observed under the electron microscope [77]. Toluene-treatment stimulates β-galactosidase activity of *E. coli* but totally destroys the galactoside permease system [73]. β-Galactosidase is not released from the treated cells, its stimulation is seen as an unmasking effect resulting from increased permeability of the membrane towards the substrate and increased accessibility of enzyme to substrate [73]. The ability of toluene to render cells permeable to small molecules has been exploited by a number of workers in recent years to study macromolecule synthesis from labelled precursors which are normally excluded from whole cells. The observation that toluene facilitates the uptake of nucleoside triphosphates [78] enabled the synthesis of cross-linked peptidoglycan to be studied in toluene-treated cells of *Bacillus megaterium* [79]. 'Toluenized' cells provide a useful wall-synthesizing system for these studies because there is minimal spatial reorientation of the membrane with respect to the wall [79]. Diethyl ether is thought to exert similar effects and studies of DNA-synthesis have been made on whole cells of *E. coli* rendered permeable to nucleotides by ether-treatment [80].

Phenethyl alcohol, $PhCH_2 \cdot CH_2 \cdot OH$, is a bacteriostatic agent [81] which is structurally-related to toluene. It inhibits DNA synthesis in *E. coli* [82] and has been used in a number of studies on DNA replication [83,84] and recombination [85]. However the effect upon DNA synthesis could be indirect, resulting from damage to the membrane in the region of attachment to the bacterial chromosome [86]. Phenethyl alcohol increases the permeability of *Neurospora crassa* to a variety of amino acids [87]; it also causes the rapid release of K^+ from *E. coli* and stimulates the uptake of acriflavine, which is not normally taken up by the cells [28]. These observations led Silver and Wendt to conclude that the primary action of phenethyl alcohol is the limited breakdown of the cell membrane [28], although recent studies suggest that the increased uptake of acriflavine is also due to damage to the permeability barrier of the outer membrane of Gram-negative cells [9].

Among Gram-positive bacteria *Bacillus cereus* and *B. megaterium* are sensitive to 0.5% phenethyl alcohol, as indicated by the rapid lysis of protoplasts and release of K^+, whereas *Streptococcus faecalis* is unaffected [51]. Direct evidence of membrane damage in sensitive cells comes from electron microscopy of thin sections which reveals changes in the geometry of the membrane profile, fracturing and solubilization of the membrane over short regions, and the occurrence of prominent, complex mesosome-like structures [51]. In a comparison of the effects of 0.25% phenethyl alcohol and toluene upon the ultrastructure of *E. coli*, Woldringh [77] showed that phenethyl alcohol generated intracellular aggregates of membrane whereas toluene caused partial dissolution of the membrane.

Although membrane damage is considered to account for the bacteriostatic

action of phenethyl alcohol in sensitive cells, studies with subinhibitory levels have shown that the phospholipid composition of *E. coli* membranes is drastically altered [88]. The changes in phospholipid composition are due to inhibition of the *de novo* rates of synthesis of phosphatidylethanolamine and phosphatidylglycerol [88]. The fatty acid composition of the phospholipids is also altered by the differential inhibition of the rates of synthesis of saturated and unsaturated fatty acids [88,89]. Studies of artificial membranes show that such changes in lipid composition alter the fluidity and permeability properties [70]. Similar effects upon the fluidity and physical state of membrane lipid have been shown in cells of *Bacillus stearothermophilus* grown at different temperatures to alter the lipid composition [90].

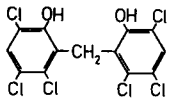
Aliphatic alcohols such as ethanol, *n*-propanol, *n*-butanol and isopentyl (isoamyl) alcohol exert disruptive effects upon bacterial membranes as shown by the lysis of protoplasts [76]. Of these, only ethanol displays any useful bactericidal activity; it is used to cleanse the skin before injections and to sterilize instruments. Butanol is used to solubilize membrane-bound enzymes [22,91], its action being similar to that of the non-ionic detergents described below.

PHENOLS

Chlorinated derivatives of phenols, cresol, and xylenol elicit the release of [^{14}C]-glutamate from *E. coli* [92]. These compounds are effective bactericides and are used in crude disinfectant and antiseptic preparations such as 'Lysol' and 'Dettol'. Their ability to denature protein is probably responsible for their disruptive action upon microbial cell membranes. In addition to damaging the membrane [92], phenol exerts a lytic action upon the cell wall; lysis occurs in growing cultures of *E. coli* [93] and is particularly noted in synchronous cultures at the point of cell division [94].

The antistaphylococcal activity of 4-*n*-alkylphenols increases with increasing alkyl chain length from 2 to 6 carbon atoms [95]. As the length of the alkyl chain increases so the water solubility falls, the lipid solubility rises, and the molecules become more surface active [95]. Penetration into the lipid-rich interior of the cell membrane therefore appears to be an important step in the antimicrobial action of the alkylphenols. 'Fattened' staphylococci with increased lipid content display some resistance to the alkylphenols because the molecules are bound to the extra lipid and are thereby denied access to the membrane [95]. Conversely, lipid-depleted cells are more sensitive than normal cells to alkylphenols and other membrane-active agents [96].

Hexachlorophene (1), tetrachlorosalicylanilide (TCS, 2), and fentichlor (3) are phenolic compounds which have been used in antiseptic soaps and shampoos,



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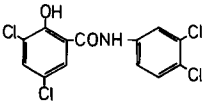

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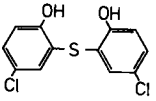


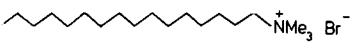
(3)

as antiperspirants, and in the treatment of skin mycoses. Hexachlorophene and TCS are now withdrawn from general use in medicated cosmetics because of toxic side effects. Fentichlor has low oral toxicity and is used internally for treatment of deep-seated skin infections. Brominated salicylanilides are used in ointments for topical application. The damaging effects of bactericidal concentrations of these compounds upon the cell membrane are shown by the leakage of intracellular components and leakage has been quantitatively correlated with antimicrobial activity [32,97,98]. Hamilton has stressed the importance of studying the biochemical effects of antimicrobial agents at cellular concentrations (expressed in molecules per bacterium) equivalent to those required to inhibit growth [7,26]. Closer examination of the effects of low concentrations of the agents reveals that hexachlorophene inhibits part of the electron transport chain [99]; TCS and fentichlor inhibit energy-dependent uptake of amino acids, and incorporation of amino acids and glucose into cellular material [26,100]; and that fentichlor is bacteriostatic at concentrations which do not induce leakage [32].

DETERGENTS

The bactericidal properties of cationic detergents have been recognized since 1935 when Domagk investigated a wide range of long-chain alkylquaternary am-

monium compounds [101]. Among the most widely-used and thoroughly-investigated of these are cetyltrimethylammonium bromide (CTAB, 4) and cetylpyridinium chloride (CPC, 5). They exhibit low toxicity and irritancy to mammalian cells, and are used as skin sterilizing agents and in antiseptic throat lozenges [102]. Their major drawback is their reduction in activity in the presence of serum or soaps, and their tendency to induce clumping of bacterial cells in suspension, a phenomenon which severely diminishes their potency [103].



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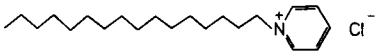

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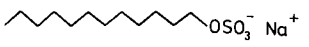


(6)

Anionic detergents such as sodium dodecylsulphate (SDS, 6) are less effective bactericides than the cationic detergents. Non-ionic detergents such as the polyethylene glycol sorbitol fatty acid esters ('Tweens') have little or no lethal effect. Although the leakage of material from cells treated with CTAB is indicative of membrane damage [104], more direct proof of the action of detergents upon the cell membrane has come from studies on the lysis of protoplasts. Gilby and Few [105] found qualitative and quantitative parallels between the bactericidal action and lysis of protoplasts of *M. lysodeikticus* produced by detergents containing a dodecyl chain attached to a charged group. The order of activity was

$$C_{12}H_{25}\text{-}NH_3^+ > \text{-}NMe_3^+ > \text{-}SO_4^- > \text{-}SO_3^-$$

Similar studies on the rate of lysis of protoplasts induced by a wide range of detergents have provided more information on their interaction with the cell membrane [106]. Cationic detergents appear to react with the phospholipid

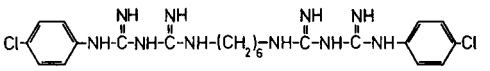
components of the membrane; the positively-charged heads associating with the phosphate groups and the alkyl chains penetrating the hydrophobic interior of the membrane. The resulting distortion of the membrane causes the protoplasts to lyse under osmotic stress but is not sufficiently drastic to cause disaggregation of isolated membranes. By contrast, anionic detergents lyse protoplasts independently of osmotic stress and disaggregate isolated membranes [104]. Their action seems to be upon the protein molecules which are solubilized by the formation of protein-detergent complexes. This property is exploited in studies on membrane protein composition where SDS is used to dissolve the proteins, and separation is achieved by electrophoresis in polyacrylamide gels containing SDS [16,107]. Non-ionic, detergents, especially (polyethylene glycol)$_{9-10}$ *p-tert.*-octylphenol ('Triton X-100') also dissociate membranes [108]. Since they are less powerful protein denaturants than SDS they are preferentially used in membrane protein studies [109], particularly in cases where enzymic activity must be retained [110]. A full account of the interaction between detergents and membranes has been given by Helenius and Simons [23].

The marked difference in bactericidal activity displayed by the three types of detergent, cationic, anionic and non-ionic, could be attributed to the role played by the cell wall. Negatively-charged components of the wall would assist the uptake of cationic molecules and repel anionic molecules. Possibly the non-ionic detergents are too large to penetrate the cell wall. Salton [104] has described the likely sequence of events following exposure of bacteria to membrane-active detergents as follows:

1. Adsorption and penetration through the porous cell wall.
2. Interaction with the membrane components leading to disorganization of the structure.
3. Leakage of low molecular weight metabolites, e.g. metal ions (especially K^+), amino acids, purines, pyrimidines, and nucleotides.
4. Degradation of proteins and nucleic acids.
5. Cell lysis following the action of wall-degrading enzymes.

The interaction of cationic detergents with microbial cells and in particular their effect upon enzymes has been reviewed by Hugo [111].

Chlorhexidine, 1,6-di(4-chlorophenyldiguanido)hexane (7) is an extremely effective bactericide which, because of its low toxicity, is widely used in medicine and veterinary practice. Its properties, applications, and mechanism of action have been discussed by Longworth [112]. The gluconate salt is freely soluble in water but aqueous solutions are only slightly surface active. To improve the wetting action, cationic or non-ionic detergents can be added without loss of bactericidal activity; however, anionic detergents are incompatible. The low toxicity and irritancy of chlorhexidine have led to its widespread use as an antiseptic wash, preventing infections in obstetrics and gynaecology [113],



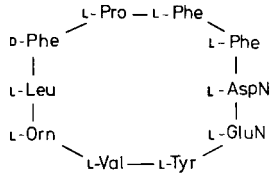
(7)

and in dentistry [114]. It is used in antiseptic creams for treatment of wounds and burns [115] and alcoholic solutions are used to cleanse the skin and hands during surgery [116]. The hydrochloride salt is less soluble in water and is used in medicated dressings, powders, and sprays [117].

Much of our knowledge of the mechanism of antimicrobial action of chlorhexidine has come from studies of its effect upon *E. coli* and *S. aureus* by Hugo and Longworth [44,47,118,119]. The effects observed depend upon the concentration employed, as is the case with most of the non-selective membrane-active agents discussed in this section. As a cationic agent, it is rapidly bound to negatively-charged groups on the cell surface [118] causing a reduction in the negative surface charge carried by the cells [119]. Low concentrations (10 to 100 $\mu g\ ml^{-1}$) produce a rapid release of cytoplasmic material from the cells [118, 120,121] which subsequently have the appearance of empty shells ('ghosts') containing no cytoplasm [47]. With increasing concentrations of chlorhexidine, the cells take up more of the drug and the rate of leakage increases. However, at concentrations above 100 $\mu g\ ml^{-1}$ there is a marked decrease in the rate of leakage [118]; the cells remain intact, although the cytoplasm appears granular and the profile of the outer region of the wall of *E. coli* is altered [27,47,119]. The reduction in leakage is due to reaction of chlorhexidine with cytoplasmic material – protein and nucleic acid – to form a precipitate which seals the cell membrane [119]. This phenomenon occurs with other cationic detergents, e.g. CTAB and polymyxin E [122], and with hexachlorophene [97]. Where chlorhexidine is used as an antiseptic the concentration is likely to be sufficient to cause gross membrane damage and massive leakage, or even coagulation of the cytoplasm. However, chlorhexidine is bacteriostatic at concentrations (around 1 $\mu g\ ml^{-1}$) which cause very little leakage, and more subtle effects must be considered to explain its inhibitory action at these low levels [112]. Studies with protoplasts of *Streptococcus faecalis* [8,123] show that chlorhexidine inhibits the membrane-bound ATPase by 50% at about 5 $\mu g\ ml^{-1}$. Energy-dependent K^+ translocation, which is thought to be coupled to ATPase in the organism, is also inhibited. Chlorhexidine appears to act directly upon the ATPase because it also inhibits the solubilized enzyme. These effects might explain the bacteriostatic action of chlorhexidine.

PEPTIDE ANTIBIOTICS

There are several groups of polypeptide antibiotics, two of which, the tyrocidins and polymyxins have effects upon bacteria similar to those produced by cationic detergents. The tyrocidin group, which includes tyrocidin A (8) and the misnamed [124] gramicidin S (9) are cyclic decapeptides containing a number of non-polar amino acids and one or two free amino groups. The left-hand portion of the decapeptide ring is common to all members of the group, and the right-hand portion is variable [125]. As described in the introduction, tyrocidin was one of the first membrane-active agents to be investigated [1,2], being a component of tyrothricin [126], a mixture of polypeptide antibiotics produced by *Bacillus brevis* [124]. Tyrocidins are more active against Gram-positive than Gram-negative bacteria; death results from general disruption of the cell membrane and leakage of cellular components [1]. The mechanism of action of tyrocidin against the fungus *N. crassa* has been studied in detail by Mach and Slayman [49]. The release into the medium of K^+, nucleotides, nucleic acids, and proteins from treated cells indicates that the membrane is severely damaged. Leakage of 260 nm-absorbing material has been characterized in greater detail.



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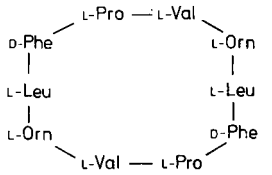

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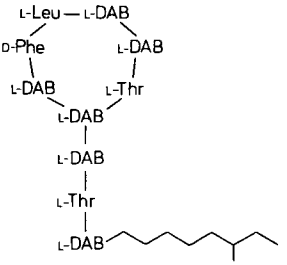
A rapid initial release, independent of temperature and occurring even at 0°, is followed by a secondary release which is absent below 12°, and much greater at 37° than 25°. It has been suggested that cell components are enzymatically degraded during the secondary phase of leakage, possibly as a result of tyrocidin-

induced damage to enzyme-containing organelles. Light microscopy and electron microscopy have confirmed the gradual depletion of cellular material brought about by tyrocidin.

The details of the molecular interaction between tyrocidin and the cell membrane are not clear. The strong surface-activity and ability to form micelles in aqueous solution are important factors in controlling the bactericidal activity. A linear analogue of tyrocidin having the same amino acid sequence is unable to form micelles and does not exhibit antibacterial activity [127]. However, gramicidin S has similar activity to tyrocidin but does not form micelles. Furthermore both the linear analogue of gramicidin S and a random copolymer made up of the same constituent amino acids exhibit greatly reduced bactericidal activity [128]. The presence of non-polar amino acids (e.g. proline, leucine, valine, and phenylalanine) and amino acids bearing basic amino residues (e.g. asparagine, ornithine, and glutamine) seems to be necessary for activity. Possibly the hydrophobic non-polar residues anchor the molecule to the membrane and the basic amino groups interfere with the ionic interactions between the membrane components. The presence of a mixture of D and L amino acids implies that the configuration of the polypeptides is also important.

The polymyxins are a group of strongly cyclic polypeptide antibiotics which includes the colistins and circulins [129–131]. They are produced by *Bacillus polymyxa* and related organisms. Polymyxins are composed of a cyclic heptapeptide moiety containing four molecules of diaminobutyric acid (DAB), one



(10)

of which bears a peptide side chain terminating in a fatty acid residue which can be 6-methyloctanoic, 6-methylheptanoic or octanoic acid [129]. Polymyxin B (10) is a typical representative of the group; it has a number of medical applications and its mechanism of action has been studied in some detail. Its structure is analogous to that of the cationic detergents: the cyclic heptapeptide, with its free amino groups, provides a positively-charged hydrophilic head, and the side

chain, with its fatty acid substituent, provides a hydrophobic tail. Aqueous solutions of polymyxin are strongly surface active, and the effects upon bacterial cells are similar to those produced by cationic detergents such as CTAB [122]. However, whereas cationic detergents tend to be more active against Gram-positive bacteria, polymyxins are chiefly active against Gram-negative organisms, including the pseudomonads, although *Proteus* species are resistant. Polymyxins are particularly useful in the treatment of *Pseudomonas aeruginosa* infections [132,133], resistant strains emerging only rarely [131,134]. They are usually administered topically, for example as a cream for application to burns [135]; kidney damage can result from systemic polymyxin therapy [133,136]. Despite adverse side reactions of this kind, polymyxins are among the very few membrane-active agents which possess sufficient selective toxicity towards microbial membranes to be of use in treating infections in man.

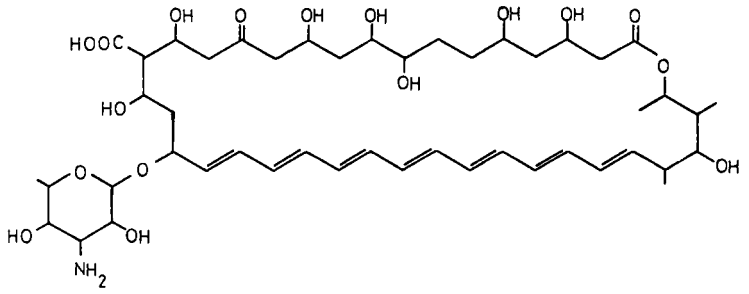
Studies by Newton [39] showed that the bactericidal action of polymyxin is due to its ability to combine with and disorganize structures which maintain the osmotic equilibrium of the cell. Rapid leakage of 260 nm-absorbing material is elicited from sensitive cells; no such leakage occurs with resistant cells [122, 137]. A more direct demonstration of the immediate increase in permeability of cells exposed to polymyxin is the uptake of a dye, *N*-tolyl-α-naphthylamine-8-sulphonic acid (TNS) with fluoresces when bound to protein [138]. Untreated cells of *P. aeruginosa* suspended in dilute solutions of TNS show no fluorescence, indicating that there are no groups on the surface with which the dye can combine. Addition of polymyxin results in immediate fluorescence due to alterations in cell permeability which allow the dye to penetrate to protein-containing regions of the cell [139]. The damage produced by polymyxin is evident from gross cytological changes, including the disappearance of nuclear material and loss of granularity of the cytoplasm [48]. Although polymyxin undoubtedly damages the cytoplasmic membrane, recent studies have concentrated on its interaction with the outer membrane of Gram-negative cells. Organisms such as *Proteus mirabilis* probably owe their resistance to polymyxin to the permeability barrier of the outer membrane, which prevents access of the antibiotic to the susceptible cytoplasmic membrane [140,141]. This subject is discussed more thoroughly in the section dealing with agents which affect the outer membrane.

The amino groups of the DAB residues in the cyclic polypeptide portion of the polymyxin molecule are necessary for the antimicrobial activity, which is reduced on acetylation [142]. Removal of the side chain from the molecule also reduces the activity by about 70% [143]. Various techniques have been used to probe the molecular interaction between polymyxin and the membrane components. Few [144] showed that polymyxin interacts with monolayers prepared from lipids and phospholipids of bacterial origin. Penetration of the monolayers was followed by an increase in surface pressure, and polymyxin E was

shown to form strong complexes with a number of microbial phospholipids, but not with lecithin (phosphatidylcholine). Hsu Chen and Feingold [145] used the polymyxin-induced release of trapped glucose marker from liposomes to study the specificity of interaction between polymyxin and phospholipids. Liposomes containing phosphatidylethanolamine were particularly sensitive to polymyxin whereas those containing *N*-methyl-substituted analogues (including phosphatidylcholine) were unaffected. The following mechanism was proposed to explain the interaction. Free amino groups on the polymyxin molecule associate with phosphate and amino groups of the polar head of phosphatidylethanolamine, and participate in a reaction involving mutual transfer of protons between the charged groups. The resulting neutralization of charge on the phospholipid upsets the balance of hydrophilic and electrostatic forces which are responsible for stabilizing the bilayer, and leads to disorganization of the liposome. Since phosphatidylethanolamine is the major phospholipid in most polymyxin-sensitive organisms [146], it is a possible site of action in the bacterial membrane [145]. However, the possibility that polymyxin reacts with other phospholipids cannot be ruled out. Using a similar technique, Imai, Inoue, and Nojima found maximum polymyxin sensitivity in liposomes containing phosphatidylethanolamine as the major phospholipid, together with small amounts of acidic phospholipid, either cardiolipin or phosphatidylglycerol [147]. Measurements of the effect of polymyxin upon the physical properties of lecithin-water dispersions indicate that the antibiotic interacts with both the polar and non-polar regions of the phospholipid [148,149].

POLYENE ANTIFUNGAL ANTIBIOTICS

The polyene group of antibiotics consists of a series of macrocyclic compounds produced by *Streptomyces* species. They are potent antifungal agents but show no antibacterial activity. The macrocyclic compounds are made up from 2 distinct parts. The first is a series of conjugated double bonds which gives the antibiotics their name. The polyene region contains from 4 to 7 double bonds; it is strongly hydrophobic and imparts rigidity to the molecule. As a consequence, polyenes are only slightly soluble in water and are light-sensitive. The carbon chain on the opposite side of the ring is fully saturated, and contains a number of hydroxyl groups which give the molecule a flexible, hydrophilic region. The amino sugar, mycosamine, is also present as a glycoside substituent in many members of the series. Amphotericin B (11) and nystatin (12) are the two polyenes which are most frequently used in clinical practice; nystatin is generally used for topical application, and amphotericin B for systemic therapy. Many other polyenes have been isolated by they are generally too toxic for clinical use.



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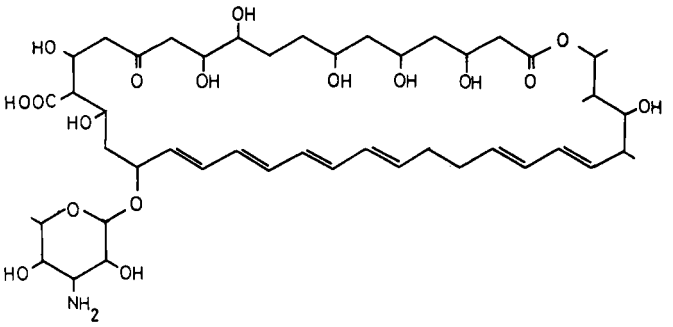

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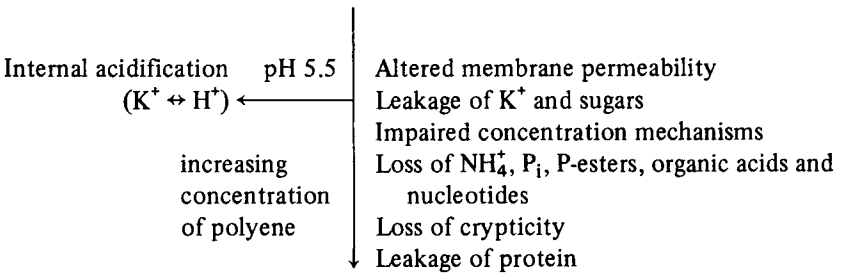
The chief use of polyene antibiotics is in the control of pathogenic and opportunistic fungal infections. In humans, mycotic infections are generally confined to the skin (athlete's foot, ringworm); to moist mucous membranes (oral thrush, candidial vaginitis); and intestinal infections. Polyenes are relatively toxic when injected into the blood stream, but they are poorly absorbed by the skin and mucosal surfaces and can be used topically. Systemic mycoses are rare but they are nearly always fatal. In such cases, the adverse side reactions which result from systemic polyene therapy, particularly kidney damage and haemolysis, are outweighed by the severity of the infection. Oral administration of the polyene, candicidin, to mammals has been suggested as a means of controlling serum cholesterol levels [150].

A number of recent reviews have described the occurrence, structure, application, and mechanism of action of polyenes [151–153]. It is interesting to relate the sequence of observations which led to our present understanding of the

interaction of polyenes with sterols in fungal membranes. Leakage of cytoplasmic constituents from yeast cells following exposure to nystatin gave the first indication of interference with membrane permeability [154]. Inhibition of certain metabolic activities concomitant with the leakage of K^+ from the cells could be reversed by addition of K^+ or NH_4^+ to the medium [155]. Binding of the polyene to the cells appeared to be an important step in the inhibitory action: yeast cells were sensitive to nystatin and bound the antibiotic even at low concentrations, whereas bacteria were insensitive and bound very little nystatin [156]. These observations suggested that sterols might be involved in the action of polyenes, since they are present in yeast cell membranes but not in bacterial membranes. This view was supported by the observation that addition of sterols to the growth medium reduced the inhibitory action of polyenes towards *Saccharomyces cerevisiae* [157]. A quantitative relationship was established between the amount of nystatin bound to various subcellular fractions of *S. cerevisiae* and the sterol content of each fraction [158]. Digitonin, an agent which forms tightly-bound complexes with certain sterols, was found to prevent binding of polyenes to fungal membranes [159]. Convincing proof came from studies of the polyene sensitivity of *Acholeplasma laidlawii.* This organism is unable to synthesize sterols but will incorporate them into its cytoplasmic membrane when they are supplied in the growth medium. Cells containing sterols in the membrane were sensitive to polyenes, whereas those with no sterol were insensitive [160,161]. On the basis of the available evidence Lampen [162] suggested the following outline of events describing the effect of polyenes upon sensitive membranes:

Polyene + cell → Membrane-polyene complex



With the development of more sensitive techniques, results were obtained which confirmed this scheme of events. Use of a K^+-selective electrode enables the release of K^+ to be accurately monitored under varying conditions, and con-

firmed that internal acidification of the cells can occur as a result of K^+–H^+ exchange [35,37,163].

Details of the molecular interactions between polyenes and membrane-bound sterols are becoming clearer as a result of experiments using various physical techniques and artificial membrane system [for reviews, see 152,153]. It is believed that when the polyene molecule reaches the cell membrane, the strong affinity of the hydrophobic region for the membrane sterol pulls the antibiotic into the membrane interior, so that the hydrophobic region lies alongside the sterol ring. This not only creates instability in the membrane by disturbing the phospholipid-sterol interactions responsible for stabilizing the membrane, but also introduces the hydroxylated region of the polyene into the membrane interior. Intrusion of the hydrophilic chain into the membrane might be sufficient to account for the observed permeability changes. It has been suggested that several sterol–polyene complexes could combine to form a pore in the membrane lined with hydroxyl groups allowing ions and small molecules to pass [67]. Detailed molecular models depicting the molecular associations which would give rise to the pores have been described [164–166]. Not all of the polyenes are capable of forming pores. Small members of the group, such as filipin, form large aggregates with sterols inside the membrane, leading to general membrane disruption [166]. The nature of the sterol component of a membrane is also an important factor in determining its polyene sensitivity. Amphotericin B and its methyl ester have greater affinities for ergosterol than for cholesterol [67,167]. This might explain why these antibiotics have a selective action against fungal membranes (which contain predominantly ergosterol) in the presence of mammalian cells (which contain mainly cholesterol), and can be used in the treatment of fungal infections of man.

INHIBITION OF ENERGY-DEPENDENT PROCESSES ASSOCIATED WITH THE CELL MEMBRANE

PROTON CONDUCTORS

In his chemiosmotic theory [52,53] Mitchell proposes that energy derived from respiratory activity, or from substrate level oxidation ('anaerobic' metabolism) produces an electrochemical potential ('protonmotive force') across the cell membrane of bacteria, mitochondria, and chloroplasts. The total protonmotive force is made up from two components: an electrical potential gradient and a chemical gradient of protons (i.e. a pH gradient) across the membrane. The protonmotive force provides the energy for the active transport of sugars and amino

acids, and the production of ATP. Agents which conduct protons across the cell membrane will dissipate the pH gradient generated by metabolism, and so reduce the total protonmotive force.

Uncoupling agents, that is, compounds which uncouple oxidative phosphorylation from respiration, have been known for many years. The most familiar is 2,4-dinitrophenol, which has been shown to promote the passage of protons across bacterial and mitochondrial membranes [168,169]. Mitchell [170] has pointed out that 2,4-dinitrophenol and many other uncouplers are weak, lipophilic acids which could dissolve in the hydrophobic interior of the membrane and act as proton conductors. Investigation of a wide range of substituted phenols showed that uncoupling activity is exhibited by phenolic compounds containing two nitro-groups [171], e.g. 2,4-dinitro-1-naphthol and 2,2'-methylene-bis(4-nitrophenol). Pentachlorophenol is also an uncoupler [172].

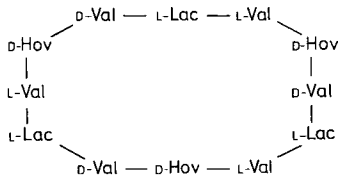
Although many uncoupling agents have been described [6] only a few of them, e.g. TCS (2) and fentichlor (3) have useful antimicrobial properties. Hamilton [26] showed that TCS inhibits a number of energy-dependent processes in *S. aureus* and *E. coli,* including the uptake of amino acids and phosphate, and the incorporation of glucose and lysine into cellular components. Harold and Baarda [172] found that TCS inhibited the energy-dependent uptake of rubidium, phosphate and certain amino acids by *S. faecalis,* but did not seriously inhibit the uptake of those monosaccharides and amino acids which did not require concurrent metabolism. Discharge of the proton gradient occurred at the same time as the inhibition of the uptake systems [56,172]. This would be expected if the proton gradient is involved in linking metabolic energy with substrate translocation as proposed in the chemiosmotic theory. Similar results have been reported for the action of bacteriostatic concentrations of fentichlor which inhibit energy-dependent substrate uptake in *E. coli* and *S. aureus* [100]. Bloomfield has confirmed that fentichlor discharges the membrane pH gradient in both organisms [54]. The presence of a membrane pH gradient in the anaerobic organism *Clostridium perfringens* has been shown by Daltrey and Hugo [173]. The gradient is completely discharged by 2,4-dinitrophenol and reduced by TCS and 4-ethylphenol, whereas other membrane-active agents, such as chlorhexidine and phenol have no effect [173].

The present state of knowledge of the coupling of energy with transport via electrochemical membrane potentials has been described by Harold [174] and by Hamilton [175]. Advances in our understanding are rapidly being made. Hugo [58] has suggested that studies on the mechanism of action of agents found to be membrane-active by the induction of leakage of cellular components, should be refined by studying their effects upon energy-linked substrate translocation systems.

IONOPHORES [175a]

The ionophores are a heterogeneous group of antibiotics which have in common the property of altering membrane permeability towards small inorganic ions. They are equally effective upon microbial and mammalian membranes and consequently are of little use in treating microbial infections in man. Nevertheless, their singular properties make them extremely valuable tools in studies of membrane transport processes. A recent review by Pressman [176] gives a thorough account of the structure, properties, and applications of ionophores.

One of the most familiar and most thoroughly studied of the ionophore antibiotics is valinomycin (13). It was originally isolated from a *Streptomyces* fermentation [177] and was found to be a potent uncoupler of oxidative phosphorylation in mitochondria [178]. Pressman recognized that, at very low concentrations (10^{-9} M) valinomycin catalysed an energy-linked K^+–H^+ exchange in mitochondria which was highly selective for K^+, Na^+ having no effect upon the exchange [179,180,181]. Valinomycin increased the electrical conductance of artificial lipid membranes by a factor of 10^8 [182] and was shown to transport K^+ ions across lipid bilayers [183] and thicker bulk lipid phases [184]. The name ionophore ('ion-bearer') was introduced for valinomycin and similarly acting compounds to indicate that they transport ions across membranes rather than form channels through which the ions can pass [184].



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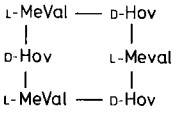

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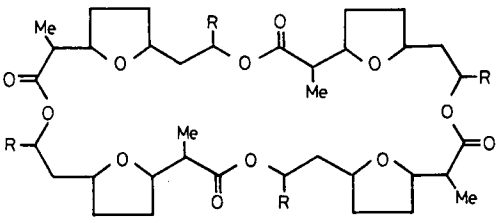

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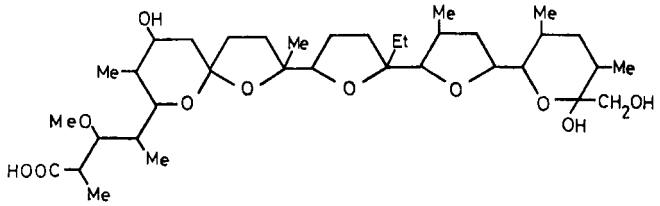
Valinomycin is a cyclic peptide containing 12 amino acid residues; 3 each of L-valine (L-Val), D-α-hydroxyisovaleric acid (D-Hov), D-valine (D-Val), and L-lactate (L-Lac) in a repeated sequence. Enniatin B (14) is a smaller cyclic peptide containing 3 residues each of D-α-hydroxyisovaleric acid and L-*N*-methylvaline (L-MeVal). Valinomycin and enniatin B contain no charged groups and are therefore classed as neutral ionophores [176]. The macrotetralide nactins (15) are also neutral ionophores, although they are of very different chemical composition. They are cyclic esters produced by various strains of *Actinomyces.* The nactin rings contain 4 alkyl substituents (*R*) and their nomenclature relates to the number of alkyl residues which are ethyl rather than methyl. Thus, nonactin contains 4 methyl groups; nonactin 1 ethyl and 3 methyl groups; dinactin 2 ethyl and 2 methyl groups, etc [185]. Carboxylic ionophores, such as monensin (16) and nigericin (17) are linear compounds containing oxygenated heterocyclic residues with a single carboxyl group on the end of the chain.



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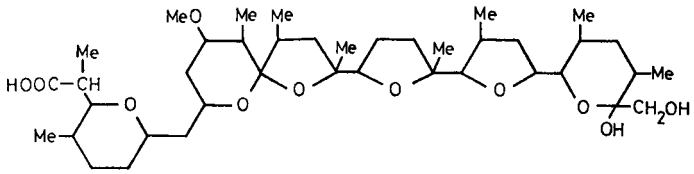

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The molecular basis of action of the ionophore antibiotics involves their ability to form lipid-soluble complexes with metal ions. Mueller and Rudin [182] suggested that the macrocyclic ionophores form coordination complexes by hydrogen bonding and dipole interactions between the cation and the carboxyl oxygen atoms of the antibiotics which project towards the centre of the ring. The carboxyl oxygen atoms of the antibiotic replace the water molecules in the hydration shell of the cation. Complexes of this type involving K^+ ions

have been observed with valinomycin [186], enniatin B [187], and nonactin [188]. The antibiotics are thought to encircle the K^+ ions in various configurations, depending upon their size and structure. The structure of valinomycin is such that, in the uncomplexed state, it has a hydrophilic and a hydrophobic side enabling it to accumulate on the surface of the membrane. On forming a complex with a K^+ ion, the hydrophilic carboxyl residues orient towards the centre of the complex, leaving the hydrophobic residues on the outside. The K^+ complex is then lipid-soluble and can pass across the membrane [189]. The precise configuration of the valinomycin molecule in the K^+ complex has been determined by X-ray crystallography [190] and NMR spectroscopy [186]. It forms a bracelet around the K^+ ion, 8 Å in diameter and 4 Å deep. Intramolecular hydrogen bonding holds the peptide chain in a configuration which allows the 6 carboxyl ester oxygen atoms to focus on the K^+ ion in the centre of the bracelet, 3 from above and 3 from below. Other metal ions have different ionic radii to K^+ and do not fit into the bracelet as neatly. Consequently valinomycin exhibits a marked preference for binding K^+; the selectivity for K^+ over Na^+ being approximately $10^4 : 1$.

Enniatin B is only half the size of valinomycin and is incapable of folding around the K^+ ion to form a bracelet. Instead it forms a planar disc complex with the K^+ ion at the centre, bound by 6 carboxyl oxygen atoms, and the lipophilic groups project outwards from the periphery of the disc [187]. This configuration has only limited ion selectivity, the $K^+ : Na^+$ ratio being less than 10 : 1. In this complex with K^+, monactin is wrapped around the central K^+ ion in a configuration likened to the seam of a tennis ball [188]. Although the carboxylic ionophores are linear compounds, they have a carboxylic acid at one end and hydroxyl groups at the other and can cyclize by head-to-tail hydrogen bonding. The monovalent metal cation complexes of monensin (16) are formed with the cyclized molecule [191]. In the case of nigericin (17), the terminal carboxyl group participates directly in binding the cation by forming an ionic bond [192].

The antimicrobial action of the ionophores is undoubtedly related to their ability to render microbial membranes permeable to K^+ ions. They induce the loss of K^+ from bacteria and, in many cases their inhibitory action towards them can be reversed by adding K^+ to the medium [189,193]. However, there is considerable disagreement over the way in which ionophores uncouple oxidative phosphorylation. The arguments concern the different concepts of the mechanisms of energy transduction and have received critical appraisal in reviews by Harold [6,174] and Hamilton [175,194].

CHANNEL-FORMING AGENTS

The gramicidins, alamethicin, and the polyenes can be termed 'quasi-ionophores' because they facilitate the passage of ions across membranes, not by carrying them in the form of lipid-soluble complexes, but by creating channels or pores in the membrane [176].

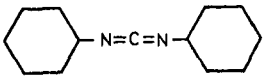
Gramicidins A, B and C are a closely related group of linear peptides in which the terminal carboxyl residue is masked by ethanolamine and the terminal amino group is formylated [195].Gramicidin S is not a member of this group, it should be classed as a tyrocidin. The gramidicins uncouple oxidative phosphorylation and increase the permeability of mitochondrial [196] and artificial lipid membranes [182] to cations. Closer examination of the effect of gramicidin A upon the electrical properties of artificial lipid bilayers shows that it produces rapid bursts of conductance across the membrane which can be explained by the rapid opening and closing of conducting channels [197]. A possible explanation of this phenomenon is that gramicidin A dimerizes in head to head association to form a spiral structure 25–30 Å long surrounding a central hole, 4 Å in diameter [198]. The structure would be long enough to span the membrane, and the pore so formed would be lined with hydrophilic groups to provide an ion-conducting channel.

Alamethicin is a cyclic peptide antibiotic containing 18 amino acid residues [199] which forms lipid-soluble cation complexes [200]. The conductance it induces in membranes varies with the applied potential and it has been suggested that a stack of 6 or more alamethicin molecules combine to form the conductance channel [201]. Alternatively, NMR studies indicate changes in the organization of the phospholipids in the membrane, each alamethicin affecting about 600 phospholipid molecules [202].

INHIBITORS OF ATPASE

ATPases are intimately involved with energy transduction in biological membranes. They catalyse the synthesis of ATP in the final stage of oxidative phosphorylation. Alternatively, they can utilize ATP to drive the translocation of Na^+ and K^+ [203]. In bacteria, ATPases are also found in association with the cell membrane, and in some cases appear as stalked particles attached to the membrane [16]. They are probably also involved in the production of ATP by oxidative phosphorylation in aerobic organisms [204,205] and in ion transport [123].

The antibiotic, oligomycin [206] indirectly inhibits mitochondrial ATPase but has no effect upon the solubilized enzyme [207]. Its action is, therefore, most likely upon some other related membrane component rather than upon the

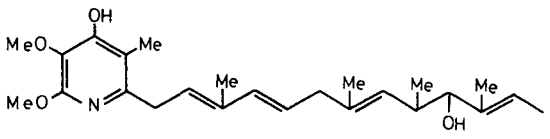


(18)

ATPase itself [6]. Oligomycin inhibits the growth of filamentous fungi and yeasts but has no antibacterial action. Dicyclohexylcarbodiimide (18) has similar effects to oligomycin upon mitochondrial ATPase. It appears to inhibit the ATPase indirectly by binding covalently to a membrane component, and has no action upon the free ATPase [208,209]. Inhibition of oxidative phosphorylation probably accounts for the action of dicyclohexylcarbodiimide upon yeasts [210] but it also inhibits the growth of *S. faecalis*, which is unable to carry out the process. Its effects upon *S. faecalis* include the inhibition of energy-dependent uptake of K^+, alanine, and phosphate; inhibition of ATP degradation but not of ATP synthesis; and inhibition of membrane-bound ATPase but not the solubilized enzyme [209]. Dio 9, an antibiotic of unknown structure, and chlorhexidine (7) might act directly upon ATPase because they inhibit both bound and free forms of the enzyme from *S. faecalis* [8,123,207].

INHIBITORS OF THE RESPIRATORY CHAIN

Piericidin A (19) is an antibiotic produced by *Streptomyces mobaraensis* [211] which has a structure resembling that of coenzyme Q. It blocks the electron transport system in mitochondria at two sites: at very low concentrations it inhibits the oxidation of $NADH_2$ and reduction of coenzyme Q; and at higher concentrations it blocks electron transport between succinate and coenzyme Q [212]. Its effects upon bacterial respiration are similar, but depend upon the organism and the nature of the electron transport system [213]; generally higher concentrations are required to bring about inhibition of bacterial respiration [214]. The antimycins are another group of antibiotics produced by *Streptomyces* species which inhibit respiratory activity in fungi and mammalian cells, but not in bacteria [215]. Hexachlorophene (1) inhibits certain cytochromes and dehydrogenases in *B. subtilis* and *E. coli* [216] and it inhibits respiratory



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activity in *B. megaterium* at a site near the terminal electron acceptor, the exact site depending upon the cytochrome composition of the membranes [99].

THE OUTER MEMBRANE OF GRAM-NEGATIVE BACTERIA

STRUCTURE AND COMPOSITION

Gram-negative bacteria are resistant to many antibiotics (e.g. erythromycin, some penicillins, rifamycin, and actinomycin D) and dyes (e.g. methylene blue and crystal violet) to which most Gram-positive bacteria are sensitive. It is generally thought that the resistance is due to the permeability barrier presented by the complex cell wall of Gram-negative bacteria [9,217,218]. The walls of Gram-positive and Gram-negative bacteria are very different in structure and chemical composition [219]. Gram-positive walls have a relatively simple structure; they are composed of peptidoglycan (40–90%) to which is covalently attached an anionic polymer, either teichoic or teichuronic acid [219,220]. Gram-negative walls are far more complex [221]. They contain a thin layer of peptidoglycan (5–20%) to which lipoprotein molecules are covalently linked [222]. The lipoproteins link the peptidoglycan with the outer membrane, a structure containing lipopolysaccharide (LPS), phospholipid, and protein. The outer membrane is a phospholipid-protein bilayer of similar form to the cytoplasmic membrane, but the LPS and lipoprotein components are asymmetrically distributed in the bilayer. The lipoprotein molecules are located on the inner face of the outer membrane, and anchor it to the peptidoglycan [222]. They might also protrude through the membrane to form pores [223,224]. The LPS molecules are located on the outer face of the membrane [225] and protrude from the cell surface into the medium in a similar manner to the glycolipids of eukaryotic organisms [226]. The LPS is capable of lateral diffusion over the cell surface [227] and the phospholipids in the bilayer form fluid domains and exhibit order-disorder transitions similar to those observed in the cytoplasmic membrane [228]. A number of representations of the structure of the Gram-negative cell wall and distribution of components in the outer membrane have been published [9,221–224, 229–232] and can be compared with the simpler structure of the Gram-positive wall [220,233–235].

PROPERTIES AND FUNCTION

Two lines of evidence support the view that the outer membrane provides a permeability barrier which makes Gram-negative cells less sensitive to many anti-

microbial agents. Firstly, treatment with EDTA releases LPS from the outer membrane and increases cellular permeability to a range of molecules that are normally excluded, e.g. actinomycin D [236] and penicillin G [237]; detergents [238]; enzyme substrates [236]; and enzymes such as lysozyme [224]. Secondly, envelope mutants of *E. coli* with incomplete LPS chains display increased uptake and sensitivity to ampicillin, penicillin G, novobiocin, nalidixic acid, chloramphenicol, gentian violet and lysozyme [239–243]. Similar, LPS-deficient (rough) mutants of *Salmonella typhimurium* are more sensitive than smooth strains towards many antibiotics and lysozyme [244]. The outer membrane appears to act as a molecular sieve with an exclusion limit of 550 to 650 daltons for saccharides which can diffuse through water-filled pores [245]. This figure contrasts strongly with the sieving properties of Gram-positive walls; the *B. megaterium* cell wall allows dextrans of molecular weights up to 100 000 daltons to pass [246]. Nikaido [218] has studied the sensitivity of a range of mutants of *S. typhimurium* with various LPS deficiencies towards a range of antibiotics and dyes. He concluded that hydrophilic antibiotics with molecular weights below 650 diffuse into the cells through aqueous pores. In contrast, hydrophobic antibiotics and dyes penetrate the cells by dissolving in the hydrocarbon interior of the outer membrane.

AGENTS WHICH DAMAGE THE OUTER MEMBRANE

Interest in the action of EDTA upon Gram-negative bacteria was stimulated by Repaske's demonstration that certain Gram-negative organisms become sensitive to the lytic action of lysozyme following treatment with EDTA in Tris buffer [247,248]. We now know that the action of EDTA is to remove part of the LPS from the outer membrane thereby giving the enzyme access to its substrate, the glycan chains of the underlying peptidoglycan [224]. Studies on the permeability changes induced by EDTA have been invaluable in determining the structure and function of the outer membrane [9]. Ca^{2+} and Mg^{2+} ions play an important part in stabilizing the outer membrane, possibly by binding between phosphate groups in adjacent LPS molecules [229]. Release of LPS from the outer membrane results from removal of the metal ions by EDTA [9]. Between a third and a half of the total LPS is released from cells of *E. coli* exposed to EDTA [249] together with some (5–20%) of the phosphatidylethanolamine and a little protein (1–2%) [250]. To achieve the maximum increase in permeability the EDTA treatment must be carried out in Tris buffer at alkaline pH. Tris buffer appears to interact with EDTA to potentiate the binding of divalent metal ions. An optimum pH and Tris concentration exists for the EDTA-facilitated action of

lysozyme on *E. coli* [248]; also the Tris cannot be replaced by similar buffers such as Tes, Hepes or Bicine [251].

The antimicrobial properties of EDTA have been described by Russell [252]. Despite its dramatic effects upon the integrity of the outer membrane, EDTA has little effect upon the viability of Gram-negative cells (except *P. aeruginosa)* and does not interfere with their transport systems [253]. Its chief value lies in potentiating the activity of other antimicrobial agents. EDTA has been shown to increase the activity of penicillin G, ampicillin, tetracycline and chloramphenicol towards *E. coli, P. aeruginosa,* and *Pr. mirabilis* [254]. *P. aeruginosa* differs from other Gram-negative bacteria in being particularly sensitive to EDTA. Cells become osmotically fragile on exposure to EDTA in the absence of lysozyme, and will undergo rapid lysis if not in an osmotically stabilizing medium [255, 256]. Addition of divalent metal ions can prevent the lysis, but the cells have decreased viability [257]. Cultures of *P. aeruginosa* tend to undergo lysis when growth is limited, and autolytic enzymes are released into the growth medium [258]. Leive has suggested that exposure of *P. aeruginosa* to EDTA might result in a cascade of events: removal of divalent metal ions from the outer membrane; release of LPS; partial autolysis and release of autolytic enzymes followed by extensive lysis of the cells [9]. The role of the cell envelope of *P. aeruginosa* in determining its resistance to antimicrobial agents; the variation in wall composition and drug resistance that can result from growth under different nutritional conditions; and the action of EDTA have all been reviewed recently [259,260].

Although the mechanism of the lethal action of polymyxins is not known with certainty, there is much evidence to suggest that the cationic antibiotics interact with anionic groups on the phospholipid molecules in the cytoplasmic membrane, and disrupt the permeability barrier [39,137]. Polymyxins also interact with components of the outer membrane in a way which is not necessarily lethal to the cells. The resistance of *Pr. mirabilis* to polymyxin B has been attributed to exclusion of the antibiotic from the cytoplasmic membrane by the outer membrane [140,141]. Although the interaction of polymyxin B with the outer membrane of this organism is not in itself lethal, it reduces the physical strength of the wall and renders the cells sensitive to the detergent, deoxycholate, Tris buffer, and to osmotic shock [261]. The wall does retain some of its differential permeability properties: the antibiotics erythromycin, actinomycin D and bacitracin; the enzyme lysozyme; and the artificial substrate triphenyltetrazolium chloride are still denied access to the cells [261]. With polymyxin-sensitive Gram-negative organisms the damage to the outer membrane produced by polymyxin B is sufficient to allow molecules the size of lysozyme to penetrate the wall [262,263] and periplasmic enzymes to be released [264].

The effects of polymyxin upon the morphology of the cell surface are quite dramatic. There have been many reports of the formation of blebs on the outer

membrane of Gram-negative bacteria following exposure to polymyxin [265–268]. The blebs are evaginations of the outermost layer of the outer membrane, and their appearance in freeze-etched specimens has been described in detail by Schindler and Teuber [269]. Bleb formation in *S. typhimurium* has been attributed to aggregation of polymyxin with LPS, cardiolipin, or phosphatidylglycerol [269]; all of which are components of the outer membrane [270] and have been shown to form complexes with polymyxin *in vitro* [271,272]. LPS appears to be the most likely component since it is thought to be located exclusively on the outer face of the outer membrane [9,225,227,229], and prior adsorption of LPS-specific phages prevents the subsequent formation of blebs with polymyxin [273]. Ultrastructural studies with *P. aeruginosa* have revealed polymyxin-induced blebs in sensitive cells but not in resistant isolates [274]. Aquisition of resistance to polymyxin correlated with changes in the architecture of the outer membrane of untreated cells as seen after freeze-etching. The surface of sensitive cells was studded with numerous, small, spherical particles which could be extracted with EDTA–Tris and were thought to contain LPS [274]. By contrast, the resistant isolates had a smooth surface. Growth in polymyxin-free medium resulted in loss of drug resistance, reappearance of the particles on the outer membrane, and bleb formation on exposure to polymyxin. The resistant isolates were also remarkably resistant to lysis by EDTA–Tris, and it seems reasonable to suppose that polymyxin and EDTA–Tris both act upon the LPS components of the outer membrane.

Similar effects of polymyxin upon wild-type (smooth) and rough mutants of *S. typhimurium* have been reported [275]. Cells of the polymyxin-sensitive, smooth, wild-type showed the familiar blebs on the outer membrane following exposure to polymyxin. Similar treatment of the rough mutants, which lacked LPS and were polymyxin-resistant, produced much smaller, flatter projections on the outer membrane. Isolated LPS forms symmetrical bilayer sheets in suspension which can be viewed under the electron microscope. Treatment of these preparations with polymyxin did not produce blebs but resulted in fragmentation of the sheets, indicating that the asymmetric distribution of LPS in the outer membrane is necessary for bleb formation [275]. Possibly, the binding of polymyxin to the LPS on the outer face of the outer membrane results in an expansion of that side of the bilayer leading to puckering of the membrane and bleb formation [269,276]. On the other hand, freeze-fracture studies on the effects of the polymyxin on the outer membrane of *S. typhimurium* indicate that both layers participate in the formation of projections [277]. The other components of the outer membrane might well contribute to the formation of blebs, but the presence of LPS seems to be the most important factor.

Chlorhexidine (7) is another membrane-active agent which has an effect upon the outer membrane of Gram-negative bacteria. Hugo and Longworth [47]

reported that at concentrations between 200 and 500 $\mu g\ ml^{-1}$ chlorhexidine produces swellings on the surface of *E. coli* which are either accumulations of drug or cellular extrusions. The failure of chlorhexidine to completely neutralize the negative surface charge carried by the cells indicates that the drug accumulates in aggregates on the cell surface rather than being adsorbed as a monolayer [119]. However, closer examination of electron micrographs has confirmed that the surface swelling, or 'blistering' of *E. coli* by chlorhexidine is associated with the outer layers of the cells [27,278]. It therefore appears that, at high concentrations chlorhexidine disrupts the structure of the outer membrane.

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3 Adriamycin and Related Anthracycline Antibiotics

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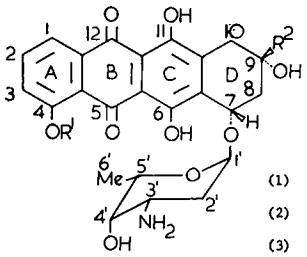
INTRODUCTION

Adriamycin * (1) and the closely related compounds daunomycin * (2) and carminomycin (3) are members of the anthracycline group of antibiotics, these being defined as antibiotics which contain a naphthacene or tetrahydronaphthacene ring skeleton [1]. Daunomycin was first isolated in 1963 simultaneously from *Streptomyces peucetius* by Farmitalia in Italy [2] and from *Streptomyces coeruleorubidus* by Rhône–Poulenc in France [3]. Subsequently the same antibiotic has been identified as a component of the rubomycin [4] and leukaemomycin [5] groups of antibiotics. Exposure of *S. peucetius* to the mutagen



(1) R^1=Me R^2=$CO^{13}CH_2^{14}OH$ adriamycin

(2) R^1=Me R^2= COMe daunomycin

(3) R^1=H R^2=COMe carminomycin

N-nitroso-*N*-methylurethane by the group at Farmitalia gave a variant organism (*S. peucetius* var. *caesius*) which was found to produce adriamycin [6]. More recently, carminomycin has been isolated, in Russia, from *Actinomadura carminata* [7,8]. The anthracyclines are cytotoxic, and adriamycin has achieved prominence in recent years as an anticancer drug with a wide spectrum of activity [9–11]. Adriamycin is of particular interest to medicinal chemists for 2 reasons. Firstly, although it differs only slightly in structure from daunomycin it has superior clinical properties. Secondly, its use is dose-limited as cardiac toxicity occurs above a cumulative dose of 450 mg m^{-2} and the incidence of cardiac toxicity increases markedly at cumulative doses greater than 550 mg m^{-2} [11]. Adriamycin is therefore a candidate lead compound for the design of new drugs. An attempt will be made here to review those aspects of adriamycin which are relevant to the design of analogues. Since adriamycin is not a unique antibiotic but is a member of the large anthracycline group of antibiotics, a discussion of other anthracyclines will be included wherever relevant.

---

* Adriamycin and daunomycin are trivial names, the international nonproprietary names are doxorubicin and daunorubicin respectively. It is the trivial names that are commonly used in the scientific literature and so these will be used throughout the review. To avoid confusion, the naphthacenequinone and tetrahydronaphthacenequinone ring systems will always be numbered, in this review, in the manner used for adriamycin although this may not always be chemically correct.

CHEMISTRY

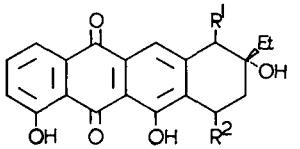
OCCURRENCE

The anthracyclines are produced by various *Streptomyces* spp. and usually occur as glycosides. The aglycones, termed anthracyclinones, are polyhydroxylated and differ in the hydroxylation pattern, the degree of saturation in ring D and the presence or absence of a carboxyl function at position 10. Reviews of the initial isolation and structure elucidation of the anthracyclinones are given by Ollis and Sutherland [12] and Brockmann [1]; known anthracyclinones are listed in *Table 3.1*, grouped according to their hydroxylation pattern. The differences in structure can be explained by considering the biosynthesis of the anthracyclinones. In 1960, ϵ-pyrromycinone (12) was shown to be derived via the acetate pathway from a starter unit of propionate followed by 9 acetate units [33] (*Figure 3.1*). More recently, daunomycinone (32), the aglycone of daunomycin, has also been shown to be formed via the same route [34], the terminal carboxyl group being lost. By inference, all anthracyclinones are polyketides and this explains the differences in hydroxylation, occasional aromatisation of ring D, loss or retention of the terminal carboxyl group and occasional substitution of a methyl group for the ethyl group at position 10 (here there is an acetate starter rather than a propionate starter). It should be noted that adriamycinone (31), daunomycinone (32) and carminomycinone (33) are unusual anthracyclinones in that the ethyl group is oxidised; methylation of the 4-OH group is a further unusual feature of adriamycin and daunomycin.

Ring D of the tetrahydronaphthacenequinone anthracyclinones contains

Table 3.1. ANTHRACYCLINONES

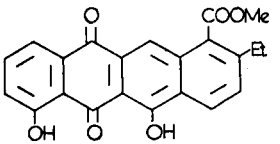
4. *4,6-Dihydroxy compounds (aklavinones* [a])
   a. Tetrahydronaphthacenequinones



R^1 = ◁ COOMe	R^2 = --- OH	aklavinone	(4)	[13]
R^1 = --- COOMe	R^2 = ◁ OH	aklavinone I	(5)	[14]
R^1 = ◁ COOMe	R^2 = ◁ OH	aklavinone II	(6)	[14]
R^1 = ◁ COOMe	R^2 = H	7-deoxyaklavinone	(7)	[15]

TABLE 3.1 (continued)

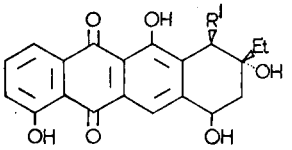
b. Naphthacenequinones



bisanhydroaklavinone (8) [16]

B. 4,11-Dihydroxy compounds (citromycinones [a])

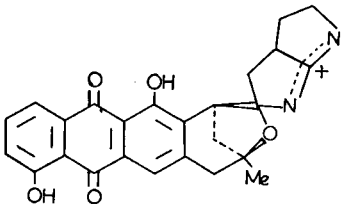
Tetrahydronaphthacenequinones



R^1=OH α-citromycinone (9) [17]

R^1 = H γ-citromycinone (10) [17]

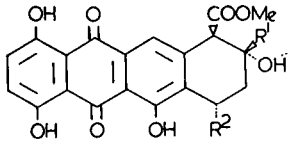
(probable structure)



aglycone of isoquinocycline (11) [18]

C. 1,4,6-Trihydroxy compounds (pyrromycinones [a])

a. Tetrahydronaphthacenequinones

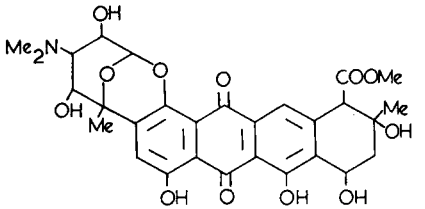


R^1 = Et R^2 = OH ε-pyrromycinone (12) [19]

R^1 = Me R^2 = OH ε_1-pyrromycinone[b] (13) [20]

(stereochemistry not proven)

R^1 = Me R^2 = H ζ-pyrromycinone (14) [1]

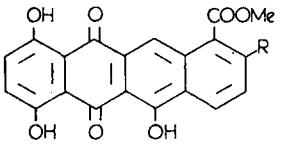


nogalarol (15) [21]

(stereochemistry not established - either 7R 9R 10S or 7S 9S 10R)

TABLE 3.1 (continued)

b. Naphthacenequinones

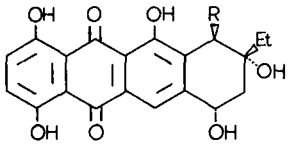


R=Et η-pyrromycinone (16) [1] [22]

R=Me η_1-pyrromycinone[b] (17) [23]

D. 1,4,11-Trihydroxy compounds (rhodomycinones [a])

Tetrahydronaphthacenequinones

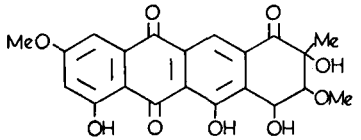


R=COOMe δ-rhodomycinone (18) [21]

R = OH α_2-rhodomycinone (19) [16]

E. 2,4,6-Trihydroxy compounds

Tetrahydronaphthacenequinones

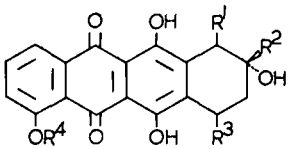


aglycone of steffimycin (20) [24]

(stereochemistry not established)

F. 4,6,11-Trihydroxy compounds (also termed rhodomycinones [a])

Tetrahydronaphthacenequinones

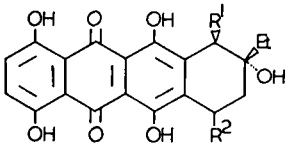


R^1 = ◁COOMe	R^2=Et	R^3=····OH	R^4=H	ε-rhodomycinone	(21) [25]
R^1 = ···· COOMe	R^2=~Et	R^3=~OH	R^4=H	θ-rhodomycinone	(22) [26]
R^1 = ◁COOMe	R^2=Et	R^3= H	R^4=H	ζ-rhodomycinone	(23) [25]
R^1 = ◁OH	R^2=Et	R^3=◁OH	R^4=H	α-rhodomycinone	(24) [27]
R^1 = ◁OH	R^2=Me	R^3=····OH	R^4=H	α_1-rhodomycinone	(25) [28]
R^1 = ◁OH	R^2=Et	R^3=····OH	R^4=H	β-rhodomycinone	(26) [29]
R^1 = ◁OH	R^2=Et	R^3= H	R^4=H	γ-rhodomycinone	(27) [29]
R^1 = ····OH	R^2=Et	R^3= H	R^4=H	10-epi-γ-rhodomycinone	(28) [29]
R^1 = ····OH	R^2=Me	R^3= H	R^4=H	β_1-rhodomycinone	(29) [27]

TABLE 3.1 (continued)

R^1=	H	R^2=Et	R^3=	H	R^4=H	10-deoxy-γ-rhodomycinone	(30) [27]
R^1=	H	R^2=$COCH_2OH$	R^3=	OH	R^4=Me	adriamycinone	(31) [6]
R^1=	H	R^2= Ac	R^3=	OH	R^4=Me	daunomycinone	(32) [30]
R^1=	H	R^2= Ac	R^3=	OH	R^4=H	carminomycinone	(33) [8]

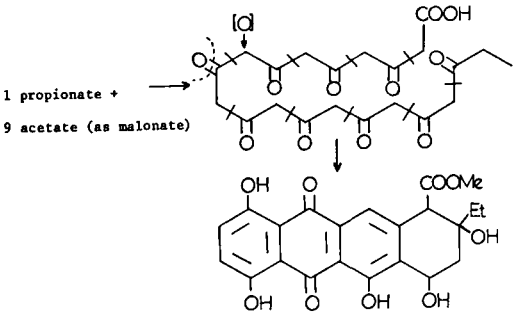
G. 1,4,6,11-Tetrahydroxy compounds (iso-rhodomycinones [a]*)*
Tetrahydronaphthacenequinones



R^1 = COOMe	R^2= OH	ε-isorhodomycinone	(34)	[31]
R^1 = COOMe	R^2 = H	ζ-isorhodomycinone	(35)	[31]
R^1 = OH	R^2 = OH	α-isorhodomycinone	(36)	[29]
R^1 = OH	R^2 = OH	β-isorhodomycinone	(37)	[29]
R^1 = OH	R^2 = H	γ-isorhodomycinone	(38)	[32]

[a] Brockmann [1] has given group names for each hydroxylation pattern, individual members are then designated by greek letters in alphabetical order with increasing R_f value. However, the system has not always been adhered to: if more than one name has been given to any one anthracyclinone, then the name consistent with Brockmann's scheme is quoted in the table.

[b] The subscript 1 has usually been used to indicate that the compound differs from the parent compound solely by substitution of the ethyl group by a methyl group.



(12)

Figure 1. Biosynthesis of anthracyclinones

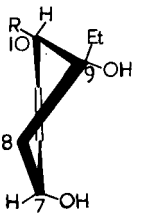
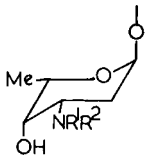


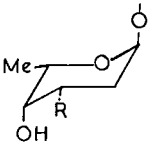
Figure 2. Conformation of ring D for a typical anthracyclinone.

Table 3.2. ANTHRACYCLINES



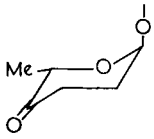
R^1=R^2=H L-daunosamine (dau) (39)

R^1=R^2=Me L-rhodosamine (roa) (41)

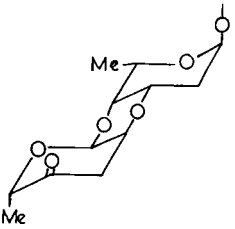


R=OH 2-deoxy-L-fucose (deofuc) (42)

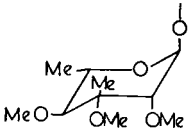
R=H L-rhodinose (rod) (43)



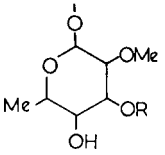
L-cinerulose A (cin A) (44)



L-cinerulose B (cin B) (45)

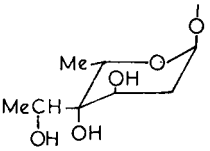


nogalose (nog) (46)



R=H (47) sugar from steffimycin

R=Me (48) sugar from steffimycin B



(49) sugar from isoquinocycline

TABLE 3.2 (continued)

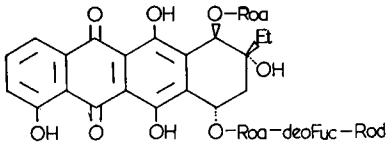
Antibiotic			*Reference*
aclacinomycin A	(50)	aklavinone [(7)roa-deofuc-cin A]	[39]
aclacinomycin B	(51)	aklavinone [(7)roa-cin B]	[39]
adriamycin	(1)	adriamycinone [(7) dau]	[6]
carminomycin	(3)	carminomycinone [(7) dau]	[8]
cinerubin A	(52)	ϵ-pyrromycinone [(7)roa-deofuc-cin A]	[40]
cinerubin B	(53)	ϵ-pyrromycinone [(7)roa-cin B]	[41]
daunomycin	(2)	daunomycinone [(7) dau]	[2]
daunosaminyl daunomycin	(54)	daunomycinone [(7) dau-dau]	[5]
doxypyrromycin	(55)	aklavinone [(7) roa]	[42]
isoquinocycline	(56)	a 7-glycoside of the aglycone (11) and sugar (49) of isoquinocycline	[18]
β-isorhodomycin II	(57)	β-isorhodomycinone [(7)roa] [(10)roa]	[43]
nogalamycin	(58)	nogalarol [(7) nog]	[21]
pyrromycin	(59)	ϵ-pyrromycinone [(7)roa]	[1]
β-rhodomycin I	(60)	β-rhodomycinone [(7) roa]	[43]
β-rhodomycin II	(61)	β-rhodomycinone [(7)roa] [(10)roa]	[43]
β-rhodomycin IV	(40)	β-rhodomycinone [(7)roa-deofuc-rod]	[43]
β-rhodomycin V	(62)	β-rhodomycinone [(7)rod$_2$,deofuc,roa] [a] [(10)rod]	[44]
β-rhodomycin S-1b	(63)	β-rhodomycinone [(7)roa-rod] [(10)roa-rod$_2$]	[45]
β-rhodomycin S-2	(64)	β-rhodomycinone [(7)roa-deofuc-rod] [(10)roa-rod$_2$]	[45]
β-rhodomycin S-3	(65)	β-rhodomycinone [(7)roa-deofuc] [(10)roa-rod$_3$]	[45]
β-rhodomycin S-4	(66)	β-rhodomycinone [(7)roa-deofuc-rod] [(10)roa-deofuc]	[45]
rhodomycin X	(67)	β-rhodomycinone [(10)roa-deofuc-rod]	[46]
rhodomycin Y	(68)	γ-rhodomycinone [(10)roa-deofuc-rod]	[46]
steffimycin	(69)	a 7-glycoside of the aglycone (20) and sugar (47) of steffimycin	[24]
steffimycin B	(70)	a 7-glycoside of the aglycone of steffimycin (20) and the sugar of steffimycin B (48)	[24]

[a] Structure of tetrasaccharide chain unknown.

asymmetric centres, and the absolute configuration of daunomycinone has been determined by degradation [30]. Comparison of the circular dichroism spectra of other anthracyclinones with that of daunomycinone led to the assignment of configuration for most of the other anthracyclinones [6,8,28,35]. The configuration at position 9 is identical for all the anthracyclinones and whereas the 7*S*,10*R* configuration is usual, the 7*R* or 10*S* configuration can occur. The

final structural consideration is the conformation of ring D. This was shown to possess the half-chair conformation by analysis of the coupling constants for the protons at C-7 and C-8 for a series of anthracyclinones [28]. This conformation, in which C-9 is out of plane with the other ring D carbon atoms, was confirmed by X-ray crystallography of γ-rhodomycinone [36] and the *N*-bromoacetyl derivative of daunomycin [37].

The anthracyclines are usually found as glycosides of the anthracyclinone with one or more deoxy sugars, glycosylation occurring at the 7 and/or 10 position. For example adriamycin (1), daunomycin (2) and carminomycin (3) are 7-glycosides of the appropriate aglycone with the sugar L-daunosamine (2,3-dideoxy-3-amino-L-fucose, 39). The following nomenclature for the anthracyclines has been suggested by Brockmann, Scheffer and Stein [38] and will be used in this review. The aglycone is stated first and then the sugar residues in square brackets with an indication of the position of substitution. Where there is a di-, tri- or polysaccharide chain, the sugar adjacent to the aglycone is quoted first, followed by the others in sequence. For example, β-rhodomycin IV (40) is



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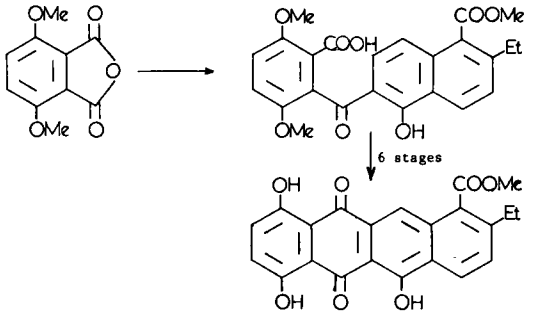
named as β-rhodomycinone[(7) roa-deofuc-rod] [(10) roa] where roa, deofuc and rod are abbreviations of L-rhodosamine (2,3-dideoxy-3-dimethylamino-L-fucose, 41), 2-deoxy-L-fucose (42) and L-rhodinose (2,3-dideoxy-L-fucose, 43) respectively. Other examples of anthracyclines, for which the full structures are known, are given in *Table 3.2.* With many anthracyclines the full structures have not yet been fully elucidated, although usually the aglycone has been identified. Examples falling in this category are the cytotetrins [47], the galirubins [48], the mycetins [1], the retamycins [49], the violamycins [50], aklavin [13], mitochromin [51] and requinomycin [52].

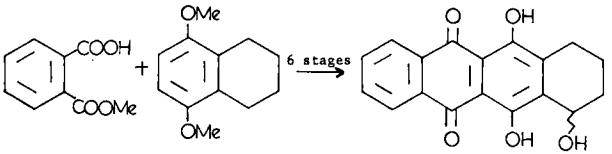
SYNTHESIS OF ANTHRACYCLINONES

Initial studies on the synthesis of anthracyclinones were concerned with structure elucidation, and centred on the preparation of naphthacenequinones. Subsequently, there has been intense interest in the synthesis of tetrahydronaphthacenequinones prompted by the usefulness of adriamycin as an anticancer



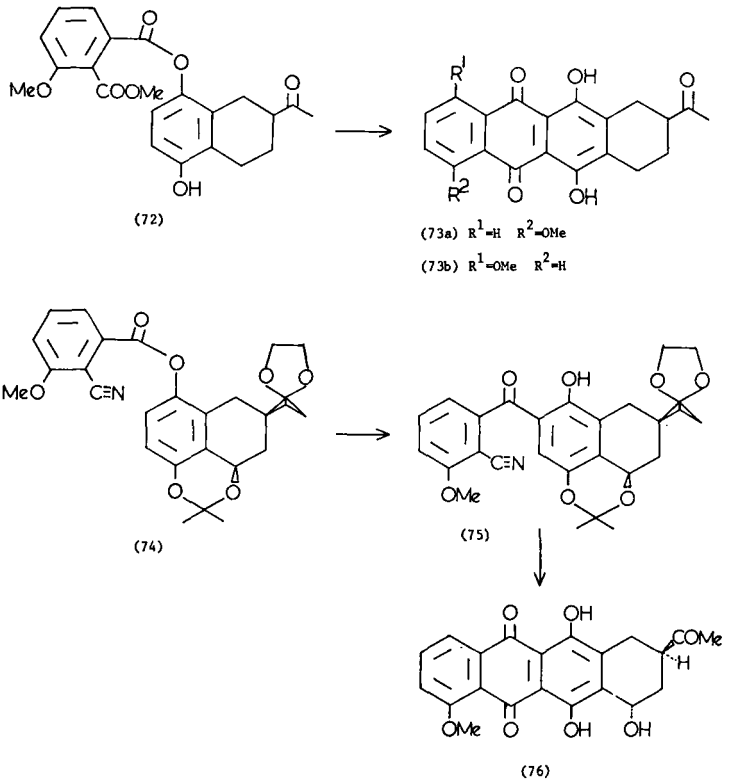
(16)

drug. The earliest syntheses of naphthacenequinones related to the anthracycline antibiotics involved Friedel–Crafts acylation of a suitably substituted naphthol with a substituted phthalic anhydride. The synthesis of η-pyrromycinone (16) by this route was the first synthesis of a natural anthracyclinone [53]. This route has also been used to prepare tetrahydronaphthacenequinones; for example (71), an analogue of daunomycinone, was prepared by acylation of dimethoxytetralin [54]. This synthesis of the ring skeleton by Friedel–Crafts acylation was also used as the basis of a 22-stage synthesis of racemic dauno-

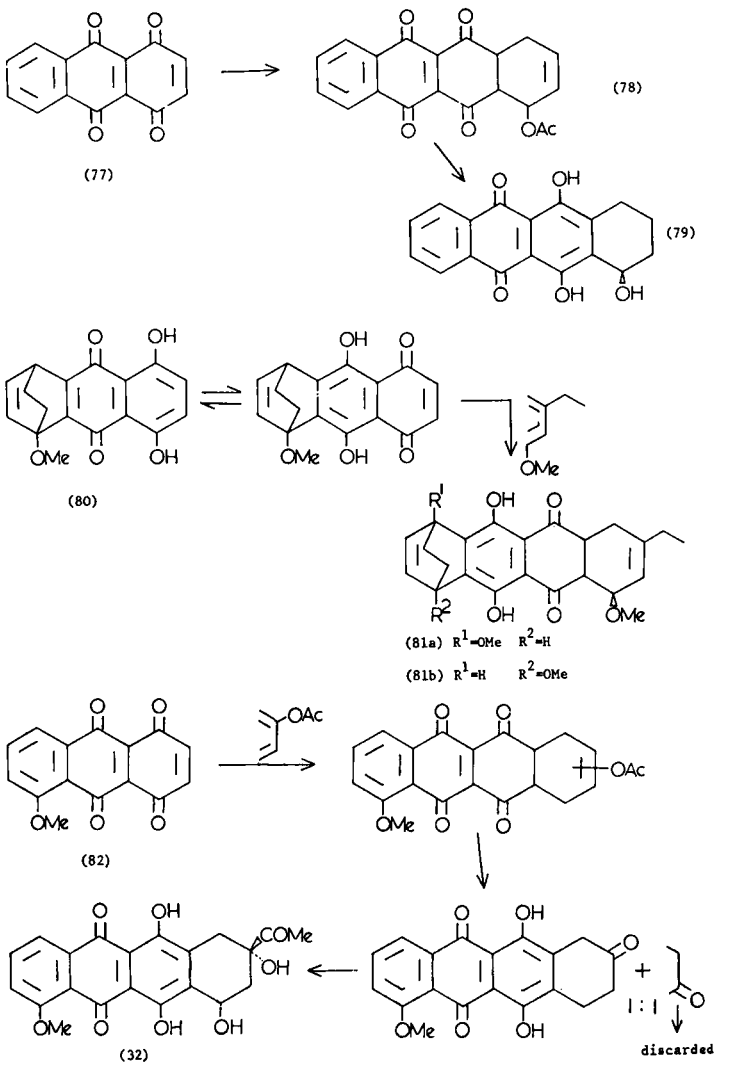


(71)

mycinone [55] and this synthetic route was further refined by Arcamone [56] to avoid racemisation and so preserve the desired stereochemistry. Nevertheless the route is complex, giving low yields of the anthracyclinone, and so the target of the most recent studies has been to derive a direct synthesis of the daunomycin or adriamycin aglycones. An alternative to Friedel–Crafts acylation for construction of the tetracyclic system is needed: with this end in mind, the Fries rearrangement was investigated. Acylation of a 5,8-dihydroxytetralin with 2-methoxycarbonyl-3-methoxybenzoic acid gave (72), but unfortunately Fries rearrangement and ring closure with BF_3-etherate was not regiospecific [57], yielding both (73a) and (73b). Regioselectivity was however achieved in the photochemical Fries rearrangement of (74) to (75), hydrolysis and cyclisation yielding 9-deoxydaunomycinone (76) [58].



Although this latter synthesis represents an elegant route to tetrahydronaphthacenequinones, the desired reduction in synthetic complexity still was not fully realised. The use of a Diels–Alder reaction to construct ring D on to a tricyclic dienophile precursor was next investigated. Quinizarin quinone (77) reacts, under suitable conditions, with 1,3-butadien-1-yl acetate to give a 7-oxygenated tetrahydronaphthacene precursor (78) which can be converted to the tetrahydronaphthacenequinone (79) [59]. However, reaction of dienes with quinizarin quinone may give an internal adduct as well as the desired end adduct, the product (end adduct or internal adduct) depending on the diene used [60, 61]. One solution is to change the dienophile so that only end adducts can be formed; (80) was therefore treated with 1-methoxy-3-methylene-1,3-pentadiene but unfortunately the product was found to contain 80% of the undesired isomer (81a) and only 20% of the desired isomer (81b) [62]. Another solution is to choose a diene which only gives an end adduct with the quinizarin quinone. Reaction with 2-acetoxybutadiene gives solely the end adduct, and this has been



used as the basis of a synthetic route which yields daunomycinone (32) in 2% overall yield from the methoxyquinizarin quinone (82) over 7 stages [63]. This reaction scheme has subsequently been extended to the preparation of $\alpha, \beta, \gamma, \epsilon$ and 10-deoxy-γ-rhodomycinones (24,26,27,21 and 30, respectively) [64] and it represents the most facile route currently described. Since daunomycinone (32) is convertible to adriamycinone (31) [65,66] then this route also represents a feasible synthesis of adriamycinone.

SYNTHESIS OF DAUNOSAMINE AND ITS ANALOGUES, AND COUPLING TO AN AGLYCONE

In order to complete a totally synthetic route to adriamycin and similar compounds, it is necessary to prepare the sugar and to glycosylate the aglycone. Synthesis of the natural enantiomer of daunosamine (39) was first reported in 1967, the reaction consisting of 10 stages and starting from L-rhamnose [67]. Subsequently an improved synthesis has been described [68] which gives an overall yield of 40% daunosamine from D-mannose over 9 stages. This latter synthesis can also be used to prepare the 3′-epimer and 3′,4′-diepimer of daunosamine [69]. A complete account of the preparation of several analogues of daunosamine has recently been given by Arcamone [56]. The final stage in the synthetic sequence is coupling of the sugar to the aglycone. The Koenings–Knorr reaction was initially used to construct the glycosidic bond in preparation of analogues of adriamycin and daunomycin from natural adriamycinone and daunomycinone. However, it is not possible to predict whether the orientation of the aglycone will be axial or equatorial, for example glycosylation of daunomycinone with a protected D-glucose or protected D-glucosamine gives the β-anomer (aglycone equatorial) [70], whereas with a protected L-daunosamine, the α-anomer (daunomycin itself) is formed stereoselectively [71]. Because of the steric variability of this reaction, alternative coupling methods have been developed at Farmitalia and these are described by Arcamone [56]. These coupling reactions complete the potential route for total synthesis of anthracyclines and in fact a totally synthetic analogue, 4′-epi-4-demethoxyadriamycin, has been reported [56]. Most analogues to date however have been prepared by semi-synthesis, or by coupling of natural daunosamine to a synthetic aglycone or by coupling of a synthetic sugar to natural adriamycinone or daunomycinone. These compounds will be discussed later when considering the effects of structural variation on biological activity.

COMMENTS

It has been shown above that there are many anthracycline antibiotics related to adriamycin and that there are feasible synthetic routes to the anthracyclines as well as there being possibilities for semi-synthetic modification. Consequently there is scope for the testing of a wide range of compounds related to adriamycin. What is needed is a rationale for selecting compounds for testing by predicting structures which might be expected to mimic the desirable properties of adriamycin. Furthermore, features of the drug responsible for unwanted effects need to be identified and eliminated. The remainder of this review will therefore

seek to delineate those features responsible for the molecular action of adriamycin, for distribution of adriamycin to its site of action and for the more favourable activity of adriamycin with respect to daunomycin. This discussion of the biological effects of the anthracyclines as a basis for the design of analogues is particularly relevant since a review of adriamycin analogues by Henry [72] showed that although a large number of compounds related to adriamycin have been tested, only rarely has any improvement in activity been seen. The majority of information on the structural requirements for activity in the anthracyclines relates to adriamycin and daunomycin, but other anthracyclines will be discussed wherever relevant information is available.

CELLULAR EFFECTS AND MOLECULAR ACTION

The anthracycline antibiotics inhibit cell growth and have a marked effect on the nuclear material which becomes non-specifically thickened, agglutinated or broken [73–75], the effects of adriamycin and daunomycin on the genetic material have recently been reviewed by Vig [76]. It is now generally accepted that an interaction with chromosomal nucleic acid is the primary lethal effect of these antibiotics although 2 main alternative theories have been suggested. The first of these proposes that a cellular electron-transport system is the site of action and the basis of this theory is the finding that both adriamycin and daunomycin, at relatively high concentration, inhibit reactions using coenzyme Q *in vitro* [77,78]. The second alternative theory is that these drugs affect membrane integrity, the evidence being an increased agglutination of cells by concanavalin A on treatment with adriamycin [79]. This increased agglutination is suggested to be due to an enhanced clustering of membrane receptors occupied by concanavalin A, this clustering being necessary before agglutination can occur. The enhanced clustering due to adriamycin requires the drug to interact with some membrane constituent, and adriamycin is known to bind to phospholipids *in vitro* [80]. The bulk of the current evidence, however, indicates that the lethal effects of these drugs result from their binding to nuclear material. This can be considered in 3 phases, entry of the drug into the cell, binding to the nuclear material, and the effect of this binding on cellular processes.

CELLULAR UPTAKE

It is important to know how adriamycin enters cells in order to incorporate the appropriate structural features in designing analogues of this drug. The current theory is that adriamycin and daunomycin passively diffuse into cells but can

be actively transported out of cells. The initial evidence for this theory was presented by Dano [81,82] who found that resistance to daunomycin in Ehrlich ascite cells was due to decreased accumulation of drug. This resistance was reversed by metabolic inhibitors and also by the Vinca alkaloids, implying that both groups of drugs are actively transported out of cells by the same active transport system which therefore has a low degree of structural specificity. Although outward movement is an active process, uptake of drug is probably (but not necessarily) passive, as there is a linear relationship between rate of entry and drug concentration in the extracellular fluid [81]. Also, daunomycin is accumulated by the cell at twice the rate for adriamycin [83,84] and an equilibrium state is reached within 1 h, whereas for adriamycin equilibrium is not reached even after 2 h [84,85]. This is consistent with a process of passive diffusion since daunomycin is more hydrophobic than adriamycin: the partition coefficients for a butanol, pH 7.4, 0.1 M buffer system are 4.1 and 2.5 for daunomycin and adriamycin respectively [84] and for an octanol, pH 7.8, 2 mM PIPES (1,4-piperazinediethanesulphonic acid) + 21 mM Na^+ buffer system are 2.8 and 0.48, respectively [86]. Further, the rate of cellular uptake of both drugs by Ehrlich ascite cells *in vitro* is reduced on lowering the extracellular pH. This is also strong evidence for passive diffusion as the method of cellular uptake since the pH partition hypothesis states that unionized drug passively diffuses across membranes far more rapidly than the less lipid soluble ionized species. The pKa of the amino group of adriamycin and daunomycin is about 8.2 [85] so the percentage of unionized drug decreases from 26% to 8% on lowering the pH from 7.8 to 6.8, leading to a markedly reduced rate of diffusion across the membrane. The cell membrane thus represents a physical barrier and there are three factors which determine the rate of uptake of adriamycin analogues. These factors are hydrophobicity (an increase will increase the rate of uptake), pKa of the amino group (a decrease will increase the rate of entry) and the pH gradient across the membrane (the lower the intracellular pH with respect to the extracellular pH, the greater the rate of entry). Drug passively diffusing reaches an equilibrium state when the concentrations of unionized drug on each side of the membrane are the same. The amount of drug accumulated in the cell then depends upon how much has been removed from the cytoplasm by binding to cellular macromolecules. Comparing the two drugs, daunomycin is accumulated to a greater extent than adriamycin since the latter has a lower rate of uptake. Adriamycin also has a lower rate of release from cells, though this is a function of the rate of release from binding sites rather than a function of the transport system [85].

BINDING TO NUCLEAR MATERIAL

Adriamycin and daunomycin bind avidly and reversibly to DNA. This interaction has been studied extensively in buffer, *in vitro*, both drugs behaving similarly. The interaction can be readily demonstrated since there is a decrease in the extinction of the drug and a bathochromic shift of about 13 nm on adding DNA to a solution of drug: also the fluorescence of the drug is markedly quenched [87–89]. Two possible mechanisms of binding can be envisaged, either intercalation of drug molecules between adjacent base pairs or binding to the outside of the helix. These mechanisms can be differentiated by considering the effect of binding on the physical properties of the drug and the DNA. When bound to DNA, daunomycin cannot be reduced polarographically [90] and does not give a bathochromic shift on basification, showing that drug molecules are removed from the aqueous environment. Also, the fluorescence of daunomycin bound to DNA is polarized when irradiated with polarized light indicating a low rate of tumbling in solution due to a rigid binding to the DNA [91]. Further, flow dichroism studies show that the chromophoric groups of adriamycin and daunomycin bound to DNA are orientated perpendicular to the helix axis [92]. All these findings strongly implicate intercalation as the primary mode of binding of adriamycin and daunomycin to DNA, and this is confirmed by considering the effect of the binding of these drugs on the properties of DNA. There is a reduction in the sedimentation coefficient of DNA and a decrease in its buoyant density in CsCl gradients on intercalation of drug due to a reduction in mass/unit length of the DNA, the viscosity of DNA solutions is also increased as a result of the reduced flexibility of the helix when drug is intercalated [88,89]. Adriamycin and daunomycin cause an increase in the T_m of DNA due to stabilization of the helix by intercalated drug molecules [93], and daunomycin is found to produce an uncoiling of circular supercoiled DNA [94] since each intercalated drug molecule causes a local untwisting of the helix. This uncoiling of circular supercoiled DNA is highly indicative of intercalation although the relevance of the degree of unwinding of the helix caused by each intercalated drug molecule to the nature of the interaction is not yet fully resolved. Finally, microcalorimetry shows the binding process to be exothermic, and the magnitude of the change in enthalpy is similar to that for the archetypal intercalating agents, ethidium and proflavine [95]. There is little doubt therefore that intercalation is the primary mode of binding of adriamycin and daunomycin to DNA. The affinity constant for the interaction is about $2–3 \cdot 10^6\ M^{-1}$ for both drugs and the maximum binding that can occur is about 2 drug molecules per 5 base pairs [89]. On saturation of the intercalation sites, further drug molecules can bind to the exterior of the helix, presumably by electrostatic interaction with the phosphate groups [96].

Where the interaction of other anthracyclines with DNA has been studied, these have been found to show broadly similar effects to adriamycin and daunomycin. Nogalamycin shows a decrease in extinction and a bathochromic shift on binding to DNA and cannot be reduced polarographically when bound; the DNA shows an increased T_m, an increased viscosity, a decreased sedimentation rate and a decreased buoyant density [92,93,97–102]. As shown above, all these effects are consistent with intercalation as the mechanism of binding of nogalamycin to DNA, further evidence is the fact that it causes uncoiling of supercoiled DNA [94]. Cinerubin also causes an increase in the T_m of DNA, an increased viscosity, a reduced sedimentation coefficient and decrease in the buoyant density of DNA [93]. The galirubins cannot be reduced polarographically when bound to DNA [90], and steffimycin B causes an increase in the T_m of DNA [24].

A model for the intercalation complex of daunomycin with DNA has been proposed on the basis of X-ray diffraction data [103]. In this model there is extensive overlap between the chromophore of the drug and the base pairs lying immediately above and below the drug. The amino sugar lies in the major groove of the DNA with its amino group interacting electrostatically with the second phosphate from the intercalation site, and there is hydrogen bonding between the 9-OH of the drug and the first phosphate from the site. This model assumes retention of the conformation of ring D which occurs in solution (see earlier). A modified version of this intercalation model based on the alternative conformation of ring D (in which the sugar is oriented equatorially with respect to ring D) is described by Henry [72]. With this model, an additional hydrogen bond is possible involving the 4′-OH group, and in the case of adriamycin the 14-OH group can also hydrogen bond to nucleic acid. In summary, the major binding force is intercalation of the planar chromophore, stabilized by an external electrostatic binding of the amino sugar. Evidence for the latter mode of binding is the reduced effectiveness of the *N*-acetylated derivative of daunomycin, as a DNA-binding agent [104]. Hydrogen bonding also contributes to the stabilisation of the complex. Potential analogues of adriamycin must therefore retain a planar hydroxyanthraquinone ring system suitable for intercalation, a basic group orientated appropriately with respect to the chromophore and should preferably also contain groups which can hydrogen bond.

EFFECTS ON CELLULAR PROCESSES

Since adriamycin and daunomycin bind strongly to DNA, chromosomal DNA is assumed to be their site of action within the nucleus, although they can also bind weakly to acidic non-histone proteins [105]. The finding that both drugs

inhibit synthesis of DNA and RNA in cell-free systems substantiates that template DNA is the site of binding particularly since the enzymic inhibition can only be reversed by excess DNA and not by additional enzyme [106,107]. In cell-free systems, adriamycin shows a slightly greater inhibitory effect than daunomycin [108]. However, it must be remembered that the cellular uptake of daunomycin is more rapid than for adriamycin, and so in cellular systems *in vitro* daunomycin shows greater activity than adriamycin [83,109,110] unless a correction is made for the difference in cellular uptake [83]. In contrast, it is adriamycin which is found to be the more potent inhibitor of nucleic acid synthesis *in vivo* (in mice) [111] and this is consistent with the greater effectiveness of adriamycin against experimental tumours in animals [112,113]. This implies that there may be a more favourable disposition of adriamycin *in vivo*. There is at present some uncertainty as to whether DNA and RNA syntheses are inhibited to the same or differing degrees [83,106,107,110,113] and this also applies to nogalamycin [114]. Both adriamycin and daunomycin also inhibit reverse transcriptase [115,116].

Considering the mechanism of the inhibition of nucleic acid synthesis, RNA synthesis is reversibly inhibited whereas inhibition of DNA synthesis is considered to be irreversible [117]. In both cases the appropriate enzyme still binds to DNA in the presence of drug [118,119] but the intercalated drug molecules appear to prevent the changes in conformation of the helix which are necessary as a preliminary to initiation of nucleic acid synthesis. On addition of drug therefore, any RNA chains in formation, for example, will be completed but no new chains will be initiated. Similarly DNA-ase II binds to DNA in the presence of drug but is unable to function [120] and as a result the drugs may prevent repair of aberrant DNA. This is probably a minor effect, the major lethal effect of adriamycin and daunomycin being their inhibition of nucleic acid synthesis. As a consequence, the drugs are more active against dividing than non-dividing cells [121] and the greatest effect is in the *S* stage of the cell cycle though inhibition in other phases of the cycle also occurs [110,122,123]. The degree and relative importance of inhibition at each stage of the cell cycle is still not fully resolved. With RNA synthesis, nucleolar RNA synthesis has been shown to be the most sensitive to daunomycin in L-1210 cells [124] and marked changes in the structure of the nucleolus are known to occur on treatment of cells with daunomycin [125].

All the effects on cellular processes, discussed above, are consistent with intercalation into cellular DNA as the molecular mode of action of adriamycin and daunomycin. The broad structural requirements for action at the cellular level have been outlined above, and it is likely that the model of the DNA complex will be refined as more information becomes available. This detailed knowledge of the interaction with DNA presents a rare opportunity for the rational

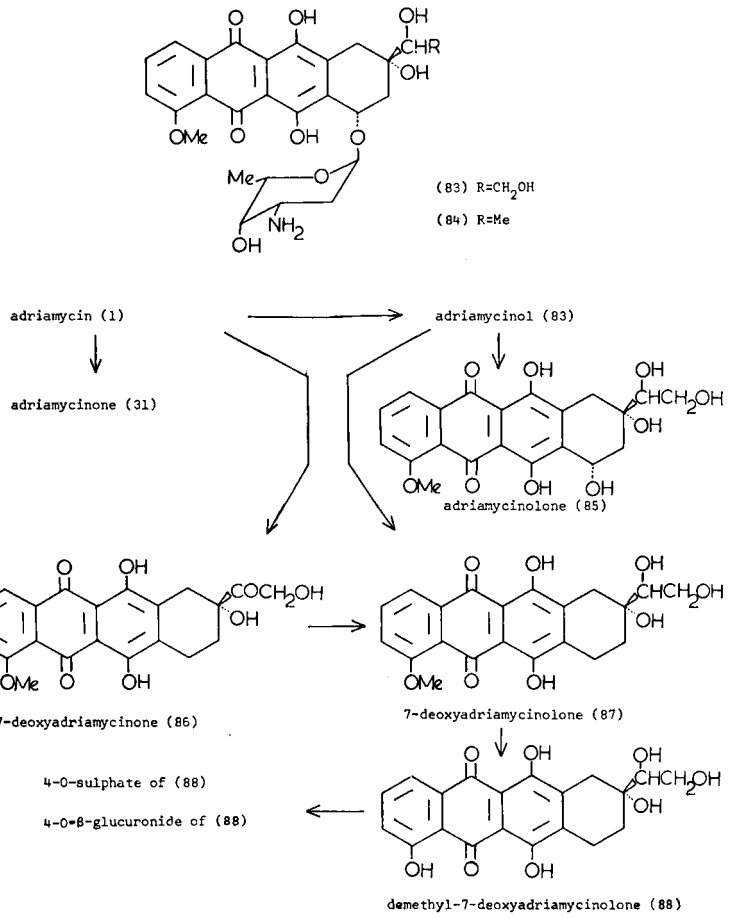
design of analogues of a drug to fit its specific receptor, although it must be remembered that the target is DNA in nucleoprotein and not the deproteinated DNA which has been used for *in vitro* studies. Also, there are some aspects of the molecular action of the anthracyclines which need to be resolved; for example, are these antibiotics base specific? Steffimycin B and nogalamycin both bind preferentially to A–T base pairs [24,98] and inhibition of nucleic acid synthesis by adriamycin and daunomycin is greatest when poly (dA–dT) is the template [106]. Furthermore, in the model suggested by Henry for the DNA–adriamycin complex, a preferential binding to A–T base pairs would be expected [72]. It is intriguing to speculate that these antibiotics bind more avidly to the important (A–T-rich) sequences than to other regions of chromosomal DNA. It is clear from the above discussion that the marked clinical advantages of adriamycin over daunomycin cannot be explained at the cellular level although it is important to realise that adriamycin has the longest retention in cells [126] (the *in vitro* half-life in cells is 12 h compared to 4 h for daunomycin [127]). A study of the data on the disposition of these drugs *in vivo* is therefore necessary to identify the chemical features responsible for the favourable *in vivo* properties of adriamycin so that these features can be incorporated in the design of analogues.

DISTRIBUTION METABOLISM AND EXCRETION

Studies of the clinical pharmacology of adriamycin and daunomycin have been bedevilled by the problems of low specificity of the assay procedures used initially and by possible degradation of drug and metabolites during extraction. However, a clear picture of the *in vivo* disposition of these drugs is now emerging. The present discussion will be primarily concerned with results obtained from human studies.

ADMINISTRATION AND METABOLISM

Neither adriamycin nor daunomycin is absorbed orally [128] due to hydrolysis in the g.i.t. and so they must be administered by i.v. infusion. Both drugs undergo metabolism [129,130] and the predominant metabolites are adriamycinol (83) and daunorubicinol (84) respectively, in which the 13-keto group has been reduced. The enzyme responsible is a cytoplasmic aldo-ketoreductase which is found in all tissues, the kidney having the highest activity [131,132]. This is not the only metabolic transformation however, both the parent drug and its 13-dihydro-metabolite are metabolised by microsomal enzymes. Most of the



information on this microsomal metabolism has been gleaned by Takanashi and Bachur from studies of the drug metabolites present in the urine of patients under treatment with adriamycin or daunomycin [133,134]. For adriamycin, about 40% of drug excreted in the urine occurs as unchanged drug and 29% occurs as adriamycinol. The remainder, the products of microsomal metabolism, are aglycones (9%) (these could be artefacts) and aglycone conjugates (22%), the metabolic transformations occurring as shown below. * The *major* metabolic pathway for adriamycin is reduction to adriamycinol (83), followed by reductive

* For convenience, the aglycones of adriamycinol and daunorubicinol will be termed adriamycinolone and daunorubicinolone, respectively.

glycosidic cleavage of the latter to 7-deoxyadriamycinolone (87) which is then demethylated and conjugated; the final 4-*O*-sulphate and 4-*O*-β-glucuronide occurring in an approximately 1 : 1 ratio. Other unidentified metabolites also occur.

The metabolism of daunomycin is qualitatively similar, but more extensive since it is a better substrate than adriamycin for the aldoketoreductase and microsomal enzymes [131]. Only 23% of the drug excreted in the urine is unchanged, 45% occurring as daunorubicinol. Although daunomycinone was not found, aglycones corresponding to (85), (86), (87) and (88) constitute 8% if the output in the urine. The remaining 24% consists of conjugates; the 4-*O*-sulphate of demethyl-7-deoxydaunorubicinolone predominating over the 4-*O*-β-glucuronide. Two further glucuronides were also found.

The metabolism of adriamycin and daunomycin is therefore extensive and is undoubtedly even more complex than our current understanding suggests. There are a number of significant points which should be noted. Firstly, the lower rate of metabolism of adriamycin may be a contributory factor to the superior *in vivo* activity of this drug over daunomycin. Secondly, it has been shown that the cellular metabolites adriamycinol (83) and daunorubicinol (84) retain activity both *in vitro* and *in vivo* [109,135]; adriamycinol is more active than adriamycin at the molecular level, conversely daunorubicinol is less active than daunomycin [84,136]. Metabolic reduction at the 13-position does not therefore inactivate the drug; in fact, it could be argued that, because of the increased hydrophilicity of the metabolites, this intracellular metabolism is desirable as it could reduce the rate of loss from the cell. In contrast, metabolism to aglycones gives inactivation [104]; these lipophilic aglycones must then be conjugated before excretion. One goal in designing analogues is to avoid production of aglycones *in vivo*. Not only would this mean that metabolic inactivation would be prevented, but also the 4-*O*-demethylation and conjugation stages would no longer be necessary as these processes merely serve to solubilise the water-insoluble aglycones. It is perhaps pertinent to note here that attempts to produce new adriamycin and daunomycin analogues by microbial conversion have only given the 13-dihydro-compounds, aglycones and 7-deoxyglycones that are produced by mammalian metabolism [137,138]. The dihydro-derivatives are in fact present in small quantities in the antibiotic-producing fermentations [5,104].

DISPOSITION AND EXCRETION

In view of the extensive metabolism of adriamycin and daunomycin it is important to distinguish between parent drug and metabolites in studies of the disposition of these drugs. This was not the case in the early studies and so care has to

be taken in appraising the results. Both drugs are rapidly cleared from the plasma following i.v. injection and are accumulated to a high degree in all tissues except the CNS [139,140]. Highest levels of drug occur in the bone-marrow, heart, lung, spleen and white blood corpuscles [141,142]. Animal studies have shown that adriamycin achieves higher peak levels than daunomycin in most tissues and persists for a longer time [143]. Elimination via the urinary route is low [141, 142]; for example one study showed that less than 6% of a dose of adriamycin was excreted in the urine over 5 days [144], the corresponding figure for daunomycin being 23% [145]. The major route of excretion is *via* the bile, again the rate of excretion being higher for daunomycin than for adriamycin [144]. A substantial proportion of the drug in the plasma is eliminated on passage through the liver, the hepatic clearance for adriamycin has been calculated to be 40% of hepatic blood flow [146]; as a consequence a reduction in dosage is required in cases of hepatic dysfunction [147]. After biliary excretion, the drug and glycosidic metabolites are probably hydrolysed in the large intestine, being excreted as aglycones in the faeces [148]. Characterisation of the pharmacokinetic profiles of adriamycin and daunomycin is complicated by the presence of metabolites, again most studies not distinguishing between parent drug and its metabolites. The curves of plasma concentration *vs.* time for the total drug (unchanged drug plus metabolites) were initially considered to be biphasic [145, 149–151] but the most recent work suggests that they are triphasic [152,153]. For unchanged adriamycin, the half-life of the first (distribution) phase is about 10 min [150,153]. The second and third (elimination) phases are protracted, the half-life of the third phase for total drug being around 30–50 h [153]. The rapid and extensive uptake into binding sites in tissue is a feature which should be mimicked in designing analogues and the tissue binding must be assumed, at this point, to be structurally non-specific. Consequently it is the physico-chemical properties of the drug which must be reproduced.

The differences in disposition of adriamycin and daunomycin are not sufficient to explain the superior *in vivo* activity of the former drug until the effects of these drugs on the immune response and on the spleen are considered. Like most anticancer drugs, adriamycin and daunomycin depress the immune response, but in animals treated with adriamycin there is a greater rejection of tumour allografts than in animals treated with daunomycin [154–156], showing the former to have a lower immunosuppressive effect. This sparing of the immune response leading to a greater host response to the tumour when treated with adriamycin than with daunomycin, is probably a major reason for the superior clinical properties of adriamycin. This is substantiated by the finding that adriamycin no longer shows its superiority in animals in which the immune response has been suppressed [157]. The reason for the difference in effects on the immune response is not yet fully resolved. It may be due to the faster rate of

accumulation of daunomycin than adriamycin by the spleen [157], and the resultant more rapid destruction of spleen cells by daunomycin [155]: the nadir of spleen cytotoxicity occurs 24 h after administration of daunomycin yet occurs 5 days after administration of adriamycin [155]. A further factor to consider is the selectivity of the drugs for different types of cells in the spleen. It appears that whereas lymphoid cells are destroyed, other cells, probably mainly macrophages, are spared [158] and as adriamycin is less toxic than daunomycin to macrophages [159], adriamycin should allow a greater sparing of these cells. This may then be the reason for its lesser immunosuppressive effect.

In summary, a consideration of the actions of adriamycin and daunomycin at the tissue level has revealed differences in the properties of these 2 drugs. Differences in the distribution to the spleen and differences in the action against subsets of the spleen cell population are probably major reasons for the superior activity of adriamycin although the lower rate of excretion and lower degree of metabolism of this drug are contributing factors. Some additions can now be made to the list of structural features to incorporate during design of adriamycin analogues. The metabolically-labile glycosidic bond must be replaced by a stable group which cannot be metabolised, in order to prevent inactivation *in vivo*. Also the physico-chemical properties of the drug must be reproduced in order to mimic the rapid distribution phase and extensive tissue binding of adriamycin. Until further evidence is available, the differences in the effects of adriamycin and daunomycin against the immune system must also be assumed to be due to differences in physico-chemical properties, emphasizing the importance of reproducing these in designed analogues. There is one final question; is it possible to overcome the toxicity of adriamycin in designing analogues?

TOXICITY

Adriamycin and daunomycin produce the toxic effects typical of drugs which inhibit nucleic acid synthesis [160–162]. All patients experience alopecia, stomatitis occurs in 70–80% of patients, depression of the bone-marrow leads to leukopenia in about 70% of patients and up to half of the patients experience nausea and vomiting [9,10,11]. Hyperpigmentation may occur [163] as may subungual haemorrhage due to activation of fibrinolysis [164]. A case of renal toxicity has been reported [165] and the limited data available suggest that these drugs are carcinogenic [166]. Also, care must be taken during administration since extravasation at the injection site can lead to severe local necrosis [167]. These side effects are manageable, for example since the nadir of myelosuppression is about 15 days after administration, the toxicity to the bone-marrow can be reduced by using a dosing interval of 3 wk [168]. The most serious toxic

effect, however, and the effect which limits the total dose, is the cardiotoxicity unique to these anthracyclines. There are 2 types of effect; firstly reversible and transient electrocardiographic changes occurring within a few hours of administration [169,170] and secondly an irreversible cardiomyopathy which can develop as late as 1–6 months after therapy and which leads to congestive heart failure [9,10,11]. Incidence of this latter fatal cardiotoxicity is increased when there is a cumulative dose in excess of 550 mg m^{-2} [83,169–172]. Although the monitoring of cardiac function can reduce the risk of cardiotoxicity [173], the ideal solution would be to identify and negate the cause of this toxic effect. There is no preferential accumulation of adriamycin or daunomycin in the heart compared with other organs, so the effect must be due to a specific sensitivity of the human heart [143]. Most animals do not respond in a similar manner and so initial studies were hampered until the rabbit was shown to exhibit a similar cardiotoxicity when treated with these drugs [174]. The changes which occur in cardiac cells include a decrease in the number of myofibrils, changes in the sarcoplasmic reticulum, degeneration of mitochondria and accumulation of sodium and potassium [168,175,176]. Although it has been suggested that mitochondrial degeneration is a secondary effect, not a cause, of cardiac toxicity [177] most studies have examined the effects of these drugs on mitochondrial respiration. A mention should first be made, however, of the suggestions that cardiotoxicity is primarily due to binding to phospholipids in cardiac cells [80], or to inhibition of DNA synthesis [178] or to a lower ability of the nucleoli of cardiac cells to recover after treatment with adriamycin or daunomycin compared with nucleoli of other cells [179]. The biochemical evidence in support of an effect against mitochondria is the inhibition by adriamycin and daunomycin of RNA synthesis in yeast mitochondria [180,181] and the direct inhibition of the electron-transport chain [182] in *in vitro* systems. The inhibition of mitochondrial respiration may therefore be due to an indirect or direct effect or by a combination of these effects [183]. It is probably the intact drug which is the mediator although the aglycones have been suggested to be responsible for the cardiotoxicity of adriamycin and daunomycin [184]. The greater toxicity to cardiac cells can be rationalized on the ground that these cells are highly dependent on mitochondrial respiration and in fact both drugs have been shown to be more effective uncouplers of oxidative phosphorylation in mitochondria from bovine heart cells than those from Ehrlich ascite cells [185]. It has been suggested recently that the transient ECG changes are due to the inhibition of mitochondrial respiration in cardiac cells and that the irreversible (fatal) cardiotoxicity is due to a cell-mediated autoimmune response [186]. This latter effect can arise as a result of inhibition of respiration causing intracellular acidosis, lysosomal action then leading to sensitisation of spleen cells followed by an auto-immune reaction [187].

There are various ways in which it has been suggested that the cardiotoxicity can be minimized; for example, avoidance of predisposing factors such as concurrent treatment with mithramycin or actinomycin or cyclophosphamide [188, 189], or irradiation [190,191]. Simultaneous administration of coenzyme Q [192] or related compounds such as tocopherol [193] is also claimed to reduce the cardiotoxicity due to the reversal of the inhibition which occurs at stages of respiration which utilize coenzyme Q. The concurrent administration of *N*-acetyl-daunomycin [194], or EDTA or ICRF-159 [195] has also been suggested, the former competing for cardiac uptake of adriamycin and daunomycin and the latter two drugs reversing cardiotoxicity presumably by a chelating effect. The cardiac glycosides might also inhibit the cardiotoxicity of adriamycin and daunomycin but this is controversial [196–200]; protagonists of the theory propose that cardiac glycosides prevent uptake of adriamycin and daunomycin since it is suggested that both drugs share the same uptake system. An alternative approach to the problem of cardiotoxicity is to attempt to direct the drug specifically to the tumour cells. Since tumour cells have a higher pinocytic activity than normal cells, they should absorb macromolecules to a greater degree, DNA has therefore undergone extensive tests as a carrier [201–203]. Covalent binding to carriers has also been examined. Daunomycin has been bound to concanavalin on the basis that concanavalin shows some preference for tumour cells [204] and daunomycin and adriamycin have been bound to tumour antibodies [205–207]. In all cases the drug retained antitumour activity. The most selective carrier suggested to date is melanotrophin. This is a peptide selectively absorbed by melanoma cells; daunomycin bound covalently to melanotrophin shows a greater activity against melanoma cells than daunomycin itself [208]. It is possible therefore to experimentally reduce the cardiotoxic effects of adriamycin and daunomycin, but the basis of the cardiotoxic effect is still unclear. In the unlikely event that aglycones are the cardiotoxic agent or that the drugs are actively absorbed in a similar manner to the cardiac glycoside, then modification of the glycoside residue as suggested earlier can eliminate these effects. If, however, inhibition of reactions using coenzyme Q or inhibition of mitochondrial RNA synthesis is the cause of cardiotoxicity, then these are features inherent in the active unit of the drug. In this case, one solution would be the selective direction of the drug to the target cell by the methods outlined above or by incorporation in liposomes containing antitumour antibodies to direct the liposome to the target [209].

EFFECT OF STRUCTURAL CHANGES ON ADRIAMYCIN ACTIVITY

In the preceding sections, the biological properties of adriamycin and daunomycin were discussed in order to identify the chemical features responsible for

activity. For molecular action, a planar hydroxyanthraquinone chromophore is required to intercalate into DNA with suitable substituents (notably the amino sugar) to stabilise the binding. On the other hand, physicochemical properties govern cellular uptake, tissue distribution and elimination. A single change in structure may affect one or both of these groups of properties, and so in discussing analogues it is important to note the test system to discern whether changes in activity reflect changes in molecular action or in distribution. A distinction will be drawn between activity and relative potency, particularly for *in vivo* studies; here activity means the increase in the survival rate of tumour-bearing mice on treatment with drug whereas relative potency compares the dose of analogue and dose of parent drug required to give the same effect. For convenience, analogues of daunomycin will be considered alongside analogues of adriamycin.

MODIFICATIONS AT C-4

Although anthracyclines containing substituents at C-1 and C-2 are known, the only adriamycin analogues with an altered A ring are modified at C-4, either by demethylation or removal of the methoxy group. No detrimental effect on the molecular action would be expected, and indeed carminomycin (3) (the 4-*O*-demethyl derivative of daunomycin) is an effective inhibitor of cellular nucleic acid synthesis, particularly DNA synthesis, and in fact is more potent than daunomycin in *in vivo* test systems [72,210]. Similarly 4-demethoxyadriamycin and 4-demethoxydaunomycin show a 10-fold increase in potency *in vivo* [56]. This is probably mainly an effect on distribution because although the complex with DNA may be slightly more stable, 4-demethoxydaunomycin shows an affinity for DNA equivalent to that of the parent drug, and it gives an equivalent inhibition of cellular nucleic acid synthesis [211].

MODIFICATIONS AT C-7

Two types of change are possible at C-7, epimerisation and modification of the substituent. Of the compounds tested to date, the 7,9-diepimer of 3-demethoxydaunomycin is the compound most closely related to 7-epiadriamycin. This 7,9-diepimer stabilises the DNA helix to melting to a lesser degree than with daunomycin and consequently it is a less active inhibitor of cell-free nucleic acid synthesis than daunomycin [211]. It is impossible to conclude whether this is due to inversion of configuration at C-7 or at C-9 or at both centres. The only other compounds for which data are available are the 2 epimers of the simplified analogue discussed earlier, compound (71). Both epimers of (71) show equiv-

alent activity as inhibitors of nucleic acid synthesis, but the epimer with the natural configuration at C-7 stabilises the helix to melting to the greatest degree. It is interesting to note that the epimer with the unnnatural configuration should not easily intercalate into DNA [72] according to the models of the adriamycin–DNA interaction discussed earlier, and further work is needed to show whether it is necessary to modify the model or whether this compound acts in a different manner. The second type of change at C-7, namely altering the substituent, will be considered in 2 stages: firstly minor changes in the daunosamine residue (mainly epimerisation) and secondly replacement of the daunosamine.

Minor changes in the daunosamine residue

(a) *C-1'*. Adriamycin is an α-glycoside, the aglycone being axial with respect to the sugar. The other anomer (1'-epiadriamycin) is found to be inactive *in vivo* [56] and this is due to an inability to bind to DNA in the same manner as adriamycin [212], presumably by steric exclusion from the intercalation site. All the β-glycosides would thus be expected to show reduced activity compared with the α-glycoside, and this is certainly the case for the β-anomer of 7,9-diepi-4-demethoxydaunomycin [211].

(b) *C-3'*. Many derivatives have been prepared by substitution at the amino group of the daunosamine residue and dramatic reductions in activity are seen. This is to be expected since a free amino group is necessary for stabilisation of the intercalation complex. A considerably weaker interaction with DNA is shown, for example, by *N*-acetyldaunomycin [87,213] and as a result, this drug requires a considerably greater concentration than daunomycin to achieve a similar inhibition of cell-free nucleic acid synthesis [213]. Other amide, urea, thiourea or peptide derivatives at this position all show decreased activity with respect to the parent drug [72,214–218]. Affinity for DNA is, however, retained when the amino group of adriamycin or daunomycin is quaternized but of course cellular uptake is much reduced, and as a result the quaternary compounds are less potent inhibitors of cellular nucleic acid synthesis [72].

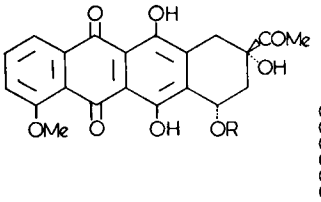
(c) *C-4'*. Compounds with an inversion of configuration at C-4' (4'-epiadriamycin and 4'-epidaunomycin) are equivalent to the parent drugs in their affinity for DNA [136], as inhibitors of cellular nucleic acid synthesis *in vitro* [219] and against tumours in animals [220]. The β-anomers (1',4'-diepiadriamycin and 1',4'-diepidaunomycin) of course show a much reduced activity in all test systems [219,220]; the distinction is however not altogether clear cut, 1',4'-diepiadriamycin is much more active than any of the other β-anomers [56]. A further point of interest is the finding that 4'-epiadriamycin has no effect on the beating rate of cultured heart cells at concentrations at which the parent drug reduces the rate [220]. Epimerisation at C-4' has therefore no deleterious effect, neither

does deletion of the 4′-OH group altogether; in fact 4′-deoxyadriamycin and 4′-deoxydaunomycin show slightly greater *in vivo* activity [221]. This argues against the involvement of hydrogen bonding of the 4′-OH group in stabilising the DNA–adriamycin complex as suggested in the model proposed by Henry [72]. *In vitro* studies with DNA should resolve this question. The 3′,4′-diepimer of adriamycin also retains *in vivo* activity but is slightly less potent than adriamycin itself [56] nevertheless demonstrating that epimerisation at C-3′ is also not detrimental.

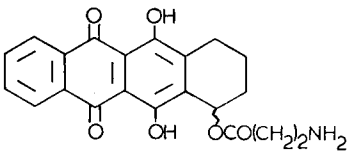
(d) *C-6′*. Initial studies with the 6′-hydroxy derivatives of the 4′-epi and 3′,4′-diepi analogues of adriamycin and daunomycin suggest that the presence of a C-6′ hydroxy group leads to a reduction in potency in *in vivo* test systems [222]. Again studies with DNA would show whether this is due to an effect on molecular action or distribution.

Replacement of the daunosamine residue

The 7-*O*-β-D-glucosamine derivative of daunomycinone was the first compound prepared in which the daunosamine residue is replaced [70]. The compound is however inactive *in vivo* [223] due to its inability to bind to DNA [213]. Esterification of the 7-OH group of daunomycin has also been attempted, for example compounds (89–93) show some activity *in vivo* [86,224]. It is interesting to compare the related compounds (94) and (95). The latter appears to have the greater degree of cellular penetration since it is about three times more active as an inhibitor of cellular nucleic acid synthesis, yet it shows no *in vivo* activity whereas compound (94) shows low activity [72]. The simpler analogue (95) therefore has an unfavourable distribution or is more rapidly hydrolysed.



(89) R = COC_6H_{10}-p-$NHCOC_6H_4$-p-OMe
(90) R = COC_6H_{10}-m-NH_2
(91) R = COC_6H_{10}-o-NH_2
(92) R = COC_6H_{10}-p-NHMe
(93) R = $COCH(NH_2)CH_2Ph$
(94) R = $CO(CH_2)_2NH_2$

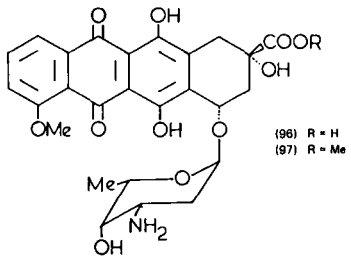


(95)

Although none of the compounds show outstanding activity *in vivo*, the retention of a low degree of activity indicates that it is possible to replace the daunosamine residue. This argues against the existence of any active transport system involving the sugar residue, and reinforces the hope that it may be possible to produce analogues which are not inactivated by metabolism to aglycone-type products.

MODIFICATION OF THE C-9 SUBSTITUENT

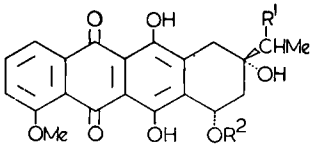
Variants at the C-9 substituent can be divided into 3 main groups, derivatives at the C-13 keto function, derivatives at the C-14 hydroxy group and compounds with an inverted configuration at C-9. The only known compounds of the latter type are 7,9-diepimers and these have already been discussed. Before considering the other 2 groups, there are 2 compounds which do not fit into either group, these are the acid (96) formed by periodate oxidation of adriamycin and the



methyl ester of this acid (97). Presence of an acidic group would be expected to hinder DNA binding, and (96) does show little stabilization of the helix to melting in contrast to (97) which stabilises the helix. Both compounds show low activity *in vivo* and a much reduced potency [225].

Derivatives at the C-13 keto function

The dihydro-derivatives adriamycinol and daunorubicinol (also called duborimycin) have already been discussed in the section on metabolism, and it will suffice to mention that these compounds retain activity *in vivo*, though potency is reduced [135]. Duborimycin is in fact being currently evaluated clinically [226]. As well as being reduced, the C-13 carbonyl group has also been treated with standard derivatising agents [72,135,213,227,228]. Compared with the parent drug, the products in general show a reduced affinity for DNA, a reduced activity against cellular nucleic acid synthesis and are generally less potent *in vivo*.



(98) R^1 = NHMe R^2 = daunosamine
(99) R^1 = $NHC_3H_6NMe_2$ R^2 = daunosamine
(100) R^1 = NHC_4H_9 R^2 = daunosamine
(101) R^1 = NHMe R^2 = H
(102) R^1 = $NHC_3H_6NMe_2$ R^2 = H

The retention of activity is expected since a substituent here would not fundamentally interfere with DNA binding according to the models proposed for the intercalation complex. Rubidazone, the benzhydrazone (*N*-benzoylhydrazone) of daunomycin [229], has been shown to be at least equivalent to daunomycin in the treatment of acute myeloblastic leukaemia [230] and in animal tests is claimed to be less cardiotoxic than adriamycin yet equivalent in potency [231]. Recently Gabbay has reported studies on a group of compounds (98–102) which have an amino substituent at C-13 [86]. All compounds bind to DNA in a similar manner to daunomycin but activity *in vivo* is much reduced as would be expected. It is significant that the water-soluble aglycones (101) and (102) retain activity in *in vitro* tests, although activity is lower than for the glycosidic analogues. This emphasises that the daunosamine unit can be replaced.

Derivatives at the C-14 hydroxyl group

Replacement of the C-14 hydroxyl group by an amino-containing substituent by reaction of 14-bromodaunomycin with, for example, morpholine or piperidine gives the expected retention of DNA-binding ability [56] though *in vivo* potency is again reduced due to distribution difficulties. Esterification of the C-14 hydroxyl group would be expected to favourably alter distribution due to an increase in lipophilicity and indeed esters of this type retain activity and potency *in vivo* [56,232]. It has been suggested that the ester group is hydrolysed intracellularly [233]; the ester unit thus might act as a transmembrane carrier. A series of esters of adriamycin in which the 3′-amino group is trifluoracetylated have also been reported and of these, *N*-trifluoroacetyladriamycin-14-valerate (AD 32) was shown to have a markedly greater activity *in vivo* than the parent drug though potency was reduced 10-fold [234]. The reduction in potency is of course expected in an *N*-acyl derivative, and in line with the previous discussion, cellular uptake of AD 32 is very rapid and there is less accumulation of drug in the nucleus compared with adriamycin [235].

COMMENTS

The results from tests on analogues of adriamycin are consistent with the hypotheses discussed earlier for cellular uptake and molecular action of adria-

mycin. The rate of cellular uptake is increased where the amino group is masked or where the compound is more hydrophobic than adriamycin. The binding to DNA is mimicked except where intercalation is precluded sterically as, for example, in compounds with the unnatural configuration at C-1′. A simultaneous change in configuration at C-7 and C-9 also hinders binding to DNA and it is important to identify which of these centres retains the natural configuration for maximum steric fit to DNA. As a result of the studies with these adriamycin analogues, it is now possible to estimate what effect structural changes may have on the cellular action of adriamycin. The problem therefore is not to design analogues which retain activity at the cellular level, but to design analogues which are metabolically stable, have a lower cardiotoxicity and yet retain potency. Although the daunosamine residue can be replaced, analogues of this type prepared to date all have a reduced potency due in some measure to a reduced affinity for DNA. Consequently, there is still considerable scope for the design and preparation of analogues of adriamycin and other anthracycline antibiotics.

CLINICAL USES

The clinical uses of adriamycin and daunomycin have recently been reviewed comprehensively by Blum and Carter [9], Skovsgaard and Nissen [10] and Carter [11], and so will only be briefly mentioned here. Daunomycin has a limited range of application, being used in the treatment of acute lymphoblastic leukaemia and acute myeloblastic leukaemia. It also has a minor use in the treatment of neuroblastoma and rhabdomyosarcoma. Adriamycin has a higher therapeutic index than daunomycin in animals [168] and although it shows no advantage over daunomycin in the treatment of leukaemias it is active against a much wider spectrum of tumours. It is of value in the treatment of lymphomas, soft-tissue and osteogenic sarcomas, paediatric malignancies such as Wilm's tumour, and adult solid tumours such as carcinoma of the bladder, breast, lung and thyroid. Both adriamycin and daunomycin are commonly used in combination with other drugs.

CONCLUSION

Adriamycin probably has the widest spectrum of activity of any current antitumour drug but its cardiotoxicity limits the total cumulative dose which can be administered; the analogues of adriamycin and daunomycin clinically evaluated to date (for example rubidazone and duborimycin) show no clear advantage over adriamycin. Considerable progress has been made in the study of the actions of

adriamycin and there is a detailed knowledge of the receptor; also the structural features required for binding to this receptor can be predicted with a high degree of confidence. Preparation of compounds which retain *in vitro* activity has not therefore been the main limitation in design of analogues. The problem is in translating *in vitro* activity into *in vivo* activity and so research on this drug is now moving into a phase where an understanding of its distribution, metabolism and excretion, as well as its mode of action, will be used as the basis for designing new analogues. Hydrophobicity and pKa are therefore key features to consider in designing analogues and it should also be remembered that it is apparently possible to replace the daunosamine unit. The use of masking groups to achieve the required distribution properties also appears feasible. Comparing daunomycin and adriamycin, the former is more rapidly accumulated by cells, but the latter has a slightly lower rate of dissociation from DNA [86] and its half-life in cells is considerably longer than that of daunomycin. Also adriamycin is metabolised and excreted at a lower rate than daunomycin and has a lower immunosuppressive effect. It is to be hoped, as more data become available on the biochemical basis of the cardiotoxicity, that it proves possible to separate the cardiotoxic and antitumour effects of this group of drugs.

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Note added in proof: Research on adriamycin continues to expand. New anthracyclines have been found e.g. rhodirubins [236], baumycins [237], musettamycin and marcellomycin [238] and 7 new compounds from the aclacinomycin-producing organism [239]. Also structures of known compounds have been elucidated e.g. aklavin [240]. The crystal structure of daunomycin has been examined [241]; in contrast to *Figure 2* (p. 131), C-8 is said to be the most out of plane atom with an H-bond between the 9-OH and 7-O functions. The most active area has been the development of synthetic routes. An anionic equivalent to the Friedel–Crafts reaction [242] and a regiospecific organometallic anthracyclinone synthesis [243] are reported but it is the Diels–Alder reaction which has been most used [244–251]. Other methods include a potential route from 2-formylhydroxyanthraquinones [252], bromination then alkylation of 2-methylanthraquinones followed by base-catalysed cyclisation [253] and Marschalk reaction of hydroxyanthraquinones [254,255].

Studies on *in vivo* properties also continue. The biliary disposition of adriamycin is reported [256] and the more lipophilic 4-demethoxydaunomycin shown to undergo some oral absorption [257]. Adriamycin gives ss and ds breaks in DNA *only* in cellular systems implying increased nuclease activity [258] although the reduced form of the drug will cleave DNA by a free radical process [259]. The free-radical semiquinone form of the drug transfers electrons to molecular oxygen, perhaps explaining the cardiotoxic effects [260], or cardiotoxicity may be due to induction of cardiac lipid peroxidation [261], inhibition of the formation (as well as function) of coenzyme Q [262] or inhibition of mitochondrial uptake of Ca^{2+} [263,264]. The protective effect of tocopherol is substantiated [260,261,265] and dextran has been evaluated as a daunomycin-carrier [266]. Cellular effects of different drugs may vary, e.g. marcellomycin causes a different type of nucleolar segregation from daunomycin [267].

Regarding analogues, the fate of AD32 in the rat [268] and its antitumour effect [269] have been reported. 9-Deacetyldaunomycin shows most activity when the 9-OH is in the 'natural' configuration [270] and 4′-epiadriamycin and 4-demethoxydanomycin show almost identical DNA-binding properties to the parent drugs [271]. It has been confirmed in cellular systems that deletion of the 4-OMe has a beneficial effect (enhanced cellular uptake): simultaneous inversion at C-7 and C-9, and inversion at $C1^1$ reduce activity [272]. New derivatives continue to be reported mainly modified at positions C-13, e.g. 13-deoxycompounds [273] or C-14 [274], the latter group includes analogues of AD32 [275].

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4 The Hypophysiotrophic Hormones

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INTRODUCTION

The role of the pituitary gland in the control and integration of endocrine activity was first realized over 50 yr ago. However, although the nervous and endocrine systems were recognised as the 2 major coordinating mechanisms of the body, some 20 yr elapsed before Harris and his colleagues demonstrated the fundamental importance of the brain, and in particular the hypothalamus, in the regulation of pituitary activity. These studies naturally led many endocrinologists to study the interrelationships of the nervous and endocrine systems and consequently neuroendocrinology is now a rapidly expanding subject. In this chapter, our current knowledge of the functional relationships between the hypothalamus and the anterior pituitary gland is reviewed.

THE PITUITARY GLAND

The pituitary gland (*Figure 4.1*) is a bilobed structure located in the sella turcica, immediately below the median eminence area of the hypothalamus. The 2 lobes are derived embryologically from different sources. The anterior lobe or adenohypohysis, which is differentiated into 3 distinct zones, the pars distalis, pars intermedia and the pars tuberalis, is derived from ectodermal tissue of the oral

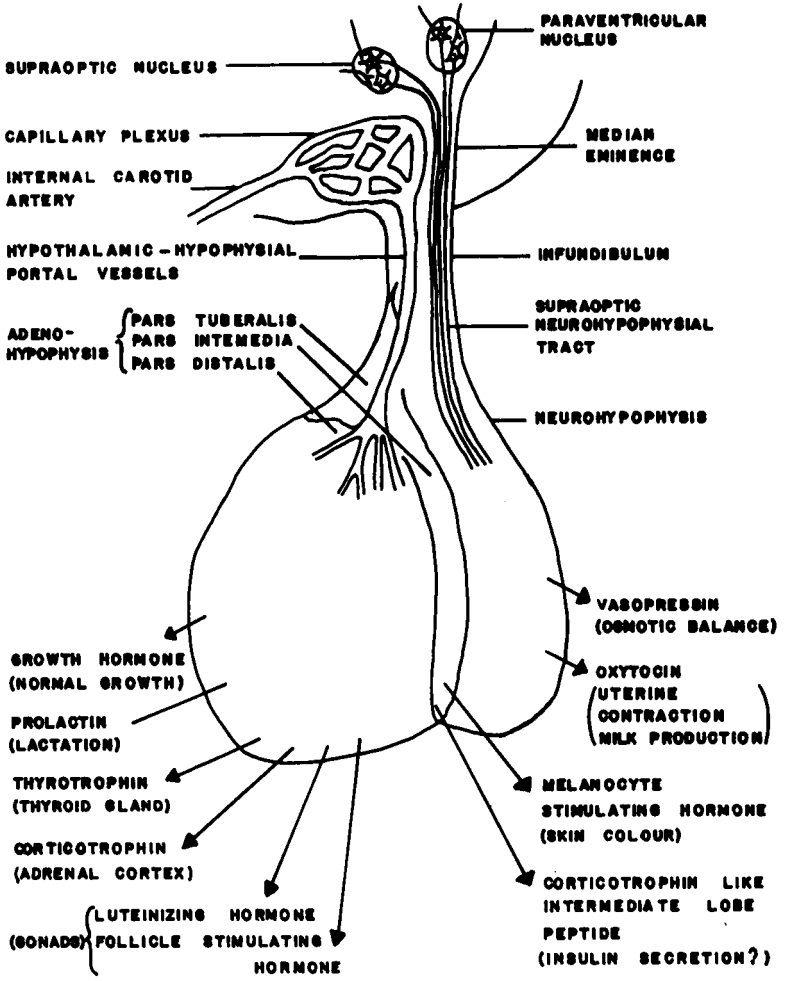

Figure. 4.1. The pituitary gland and its anatomical relationships with the hypothalamus [1a]

epithelium. The posterior lobe or neurophophysis is formed from nervous tissue of the diencephalon.

Although the adenohypophysis receives few, if any, nerve fibres, it is linked to the median eminence area of the hypothalamus by a highly specialized portal vascular system from which it receives its entire blood supply. The hypothalamus is supplied with blood by a branch of the internal carotid artery which forms a capillary plexus, the primary plexus, within the median eminence. The blood flows into the hypothalamo-hypophysial portal vessels which pass down the infundibulum to the adenohypophysis where they break into sinusoidal vessels. The nervous and vascular supplies of the neurohypophysis differ markedly from those of the adenohypophysis. The neurohypophysis is well supplied with nerve fibres, being connected to 2 prominent groups of cells in the hypothalamus, the supraoptic and paraventricular nuclei, by the well-defined supraoptico-hypophysial tract. There is no vascular link between the hypothalamus and the neurohypophysis but the latter is supplied directly by a pair of posterior hypophysial arteries which originate from the internal carotid arteries.

The anterior pituitary gland is responsible for the synthesis and release of at least 8 hormones of which the physiological actions are well understood. The secretions of the pars distalis include growth hormone (normal growth and development), thyrotrophin (thyroid gland activity), corticotrophin (maintenance of the adrenal cortices), prolactin (lactation), luteinizing hormone (LH) and follicle stimulating hormone (FSH) (gonadal function), while the pars intermedia produces melanocyte stimulating hormones (α-MSH and β-MSH) (camouflage in lower vertebrates). Recent evidence suggests that the pars intermedia also secretes a substance similar to corticotrophin, the corticotrophin-like intermediate lobe peptide (CLIP), which may be associated with the control of insulin secretion. With the exception of 3 glycoproteins (Thyrotrophin, LH and FSH), the hormones of the adenohypophysis are polypeptides. Each of the hormones is synthesized by specific cell types randomly distributed throughout either the pars distalis or pars intermedia.

The posterior pituitary gland secretes only 2 hormones, each of which is an octapeptide: vasopressin, the antidiuretic hormone, is concerned with osmotic balance and oxytocin with mammary milk production and uterine contractions.

EVIDENCE FOR HYPOTHALAMIC CONTROL OF ANTERIOR PITUITARY FUNCTION

When Harris [1] and Brooks [2] first suggested, perhaps rather tentatively, that the adenohypophysis may be controlled by humorally transmitted stimuli from the hypothalamus, little interest was expressed in their hypothesis. This was

probably mainly because it was thought at that time that the direction of blood flow in the hypothalamo-hypophysial portal vessels (which form the only anatomical link between the hypothalamus with the anterior pituitary gland) was from the adenohypophysis to the hypothalamus. The direction of flow in these vessels subsequently became a matter of controversy and it was not until 1949, after the development of an elegant technique for the direct observation of blood flow in the portal vessels of rats, that Green and Harris [3] were able to state dogmatically that the blood passes from the hypothalamus to the pituitary gland.

It is now firmly established that the synthesis and release of the hormones of the adenohypophysis are controlled by substances released from nerve endings in the hypothalamus and conveyed to the anterior pituitary gland via the portal vessels. The evidence which demonstrated the fundamental importance of the hypothalamus and the hypothalamo-hypophysial portal vessels came mainly from experiments which involved transection of the pituitary stalk, transplantation of the pituitary gland to a site remote from the sella turcica or electrical stimulation of the hypothalamus.

Transection of the pituitary stalk in rabbits is followed by gonadal atrophy [1] and subsequent recurrence of oestrus [2]. Histological studies demonstrated that, unless a wax-paper plate is inserted between the 2 cut ends, transection of the stalk is followed, almost invariably, by regeneration of the portal vessels and, that the degree of regeneration is correlated with the degree of restoration of pituitary activity [4].

The simple experiment of removing the pituitary gland from the sella turcica to another site of the body also demonstrated the role of the hypothalamus in the control of anterior pituitary function. When the transplanted tissue is placed in a site remote from the sella turcica, for example, the anterior chamber of the eye, the kidney capsule or temporal lobe of the brain, partial or complete atrophy of the ovaries, testes, reproductive tracts, thyroid gland and adrenal cortices is observed together with a reduction in the rate of body growth. When, however, the transplanted tissue is placed in the vicinity of the hypothalamus and pituitary stalk, regeneration of the hypophysial portal vessels occurs and anterior pituitary function is restored as shown by the maintenance of reproductive, thyroid and adrenocortical activity [5].

As early as 1936, it was realised that diffuse electrical stimuli applied to the head or lumbar spinal cord of rabbits [6] or rats [7] enhance adenohypophysial activity. In an attempt to delimit the neural structures involved, closely localised electrical stimuli were applied directly to regions of the hypothalamus and anterior pituitary gland of anaesthetised animals. Electrical stimulation of the pituitary gland was ineffective in causing gonadotrophin release but such stimulation was fully effective when applied to discreet areas of the hypothalamus

[1,8,9]. Corresponding lesions in the hypothalamus were, however, not always effective in suppressing pituitary activity. Moreover, since the electrical stimulation of the hypothalamus was performed in anaesthetised animals, the possibility existed that the endocrine activity observed was the result of a complication induced by the anaesthesia.

Harris [10] believed it important to study endocrine function in conscious animals and, accordingly, developed an ingenious method for 'remote control stimulation' of the hypothalamus in unanaesthetised rabbits. Using this technique, experiments were performed for relatively long periods without concomitant operative trauma. Furthermore, the experiments were repeated many times in the same animal, thereby reducing the chances that variable factors, such as differences in the nutritional or oestrous state of the animal, influenced the result. The method involved a preliminary operation in which a small flat coil was inserted between the skull and scalp. The inner turn of the coil was connected to an electrode implanted in the hypothalamus and the outer turn of the coil to a second electrode, the indifferent electrode. Stimulation of the hypothalamus was readily achieved by placing the animal's head in an electromagnetic field and inducing a voltage in the buried coil. The experiments which followed demonstrated clearly that stimulation of discreet areas of the hypothalamus increases markedly the activity of the reproductive system [11] thyroid gland [12] and adrenal cortex [13]. It was these studies, which are now regarded as a major landmark in endocrinology by biologists throughout the world, that led Harris to postulate, quite correctly, that the hypothalamus liberates chemical transmitter substances into the hypophysial portal vessels which are carried to the anterior pituitary gland to exert a specific influence over the activity of the gland.

THE CHEMISTRY, ACTIONS AND CONTROL OF THE HYPOPHYSIOTROPHIC HORMONES

The realisation that the hypothalamus contains substances capable of affecting pituitary activity naturally led to attempts to isolate and identify these neurohormones. It seemed unlikely that the traditional chemical transmitters of the parasympathetic and sympathetic nervous system, acetylcholine and noradrenaline, were responsible since neither influenced adenohypophysial activity when injected directly into the pituitary gland. Histamine and adrenaline were also ineffective in this respect despite their being present in considerable quantities in the hypothalamus. Attempts to separate the hypothalamic hormones, or 'releasing hormones' as they are known, has therefore primarily involved chemical extractions of the hypothalamus. This laborarious work, which has been

carried out mainly in the laboratories of Schally and Guillemin, has not been without problems. Since the hypothalamus contains only minute quantities of each releasing hormone, extractions are performed on large numbers of pooled hypothalami. The yields from each extraction are usually very low and the 'pure hormone' fractions, which are generally peptide in nature, sometimes contaminated and unstable. Nevertheless, several releasing hormones have now been isolated by chemical extraction techniques, and their structures determined. It was originally postulated that each pituitary hormone is controlled by a single hypothalamic neurohormone but it now appears that the system is more complex. A pituitary trophic hormone may be under both excitatory and inhibitory influences from the hypothalamus and conversely a hypothalamic neurohormone may activate more than one pituitary cell type. Attention has also been focussed on the mechanisms which control the production of the hypothalamic hormones. The methods have involved primarily electrical stimulation or lesions of discrete areas of the brain, implantation of pharmacological agents into specific brain regions or *in vitro* techniques. None of these methods may be used without criticism. Lesions in specific regions may result in tissue damage to other areas. The doses used in implantation studies may be too high and the possibility of non-specific diffusion to other areas cannot be disregarded. Moreover, the results obtained from artificially induced situations *in vitro* may not be of any physiological significance. However, despite these limitations, there is often good agreement between the results obtained using different experimental procedures and knowledge concerning the control of the hypophysiotrophic hormones is rapidly advancing.

It appears that many parts of the brain contribute to the influx of information which controls the synthesis and release of the hypothalamic hormones. The important brain structures in this regard are believed to be the septum, hippocampus, anterior thalamus, amygdala, piriform cortex and midbrain although additional areas may influence hypothalamic activity indirectly. It appears that the production of a single hormone involves the integration and differentiation of a barrage of afferent impulses, which may be either excitatory or inhibitory, from several regions of the brain. The chemical nature of the hypothalamic neurones which influence the activity of the releasing hormone cells is not yet fully understood. Many monoaminergic and cholinergic pathways can be traced through the hypothalamus and studies both *in vivo* and *in vitro* indicate that several neurotransmitter substances may influence the production of each hypothalamic hormone.

In addition to influences from higher centres of the brain, negative feedback mechanisms play an important part in the regulation of the secretion of certain hypothalamic and pituitary hormones. The evidence in the literature suggests that not only the target organ hormones are important in this respect but that

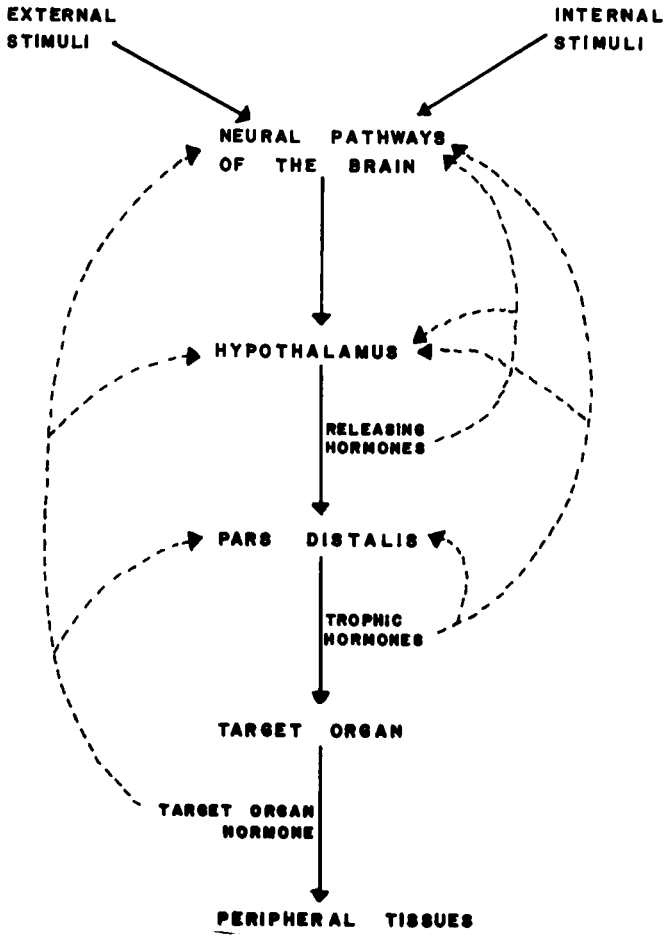

Figure 4.2. Brain-pituitary-target organ control mechanisms [1a]

the pituitary hormones and possibly the hypophysiotrophins themselves may exert inhibitory influences on the functional activity of the hypothalamo-hypophysial complex (*Figure 4.2*).

In the sections which follow the chemical nature, biological actions and control of each of the hypophysiotrophic hormones is considered.

THYROTROPHIN-RELEASING HORMONE (TRH)

Although Greer [14] demonstrated clearly that hypothalamic lesions impair the secretion of thyrotrophin (TSH) from the pituitary gland, some years elapsed before the thyrotrophin-releasing activity of hypothalamic tissue was described. A Japanese team [15] showed that anterior hypothalamic extracts stimulate the release of TSH *in vivo* but their studies were harshly criticized and one group of workers [16] was unable to repeat the findings using extracts from the same source. Schreiber [17] observed that hypothalamic extracts increase the acid phosphatase activity of pituitary tissue *in vitro*. He noted a correlation between pituitary acid phosphatase and thyroid activities and postulated that the increase in acid phosphatase activity was a manifestation of the thyrotrophin releasing activity in the hypothalamic extracts. The ability of such extracts to release TSH from pituitary tissue *in vivo* and *in vitro* was subsequently convincingly demonstrated [18–20]. Furthermore Schreiber's group were able to evoke TSH release by applying 'purified hypothalamic extracts' to hypophysial grafts in the anterior chamber of the eye and to pituitary tissue *in vitro*.

The initial purification of TRH from hypothalamic tissue involved gel filtration on Sephadex G-25 columns followed by chromatography [19]. Guillemin [21] found the active substance to be heat-stable but readily inactivated by proteolytic enzymes and suggested that TRH is a small polypeptide. It was first successfully isolated from porcine hypothalami [22]. An outline of the procedure is as follows: Fragments of porcine hypothalamic tissue containing essentially pituitary stalk and median eminence were dehydrated by lyophilization. The lyophilized fragments were defatted and extracted in acetic acid before undergoing gel filtration on Sephadex G-25. The active fractions were concentrated, desalted by phenol extraction and subjected firstly to ion-exchange chromatography on a column of carboxymethylcellulose and subsequently to countercurrent distribution in a system of 0.1% acetic acid : butanol : pyridine (11 : 5 : 3 v/v). The TRH obtained was further repurified by free-flow electrophoresis and partition chromatography. It was effective *in vivo* in nanogram quantities and released 200–2000 times its own weight of TSH [23,24]. After acid hydrolysis purified TRH was shown to contain 3 amino acids (histidine, glutamic acid and proline) in equimolar ratios [22]. The structural sequence was determined by the Edman procedure to be Glu-His-Pro. However, even at relatively high dose levels, various synthetic isomeric tripeptides containing these amino acids (including Glu-His-Pro) failed to influence TSH release [23,25]. The lack of activity of Glu-His-Pro was readily explained by studies indicating that TRH possesses neither a free amino or carboxy group. Bøler, Enzmann, Folkers, Bowers and Schally [26] subsequently characterized TSH as (pyro) Glu-His-Pro (NH_2). They also successfully synthesized the peptide and showed that the

synthetic compound possessed the same biological activity as the natural hormone. Burgus, Dunn, Desiderio and Guillemin [27] using ovine material reached conclusions similar to those of Bøler and colleagues and the tripeptide nature of TRH was finally confirmed [28,29]. TRH has now been isolated from a variety of mammalian species and, as yet, no species differences have been described. However, it is perhaps interesting to note that mammalian TRH does not influence TSH secretion in some amphibian species [30].

TRH is synthesized in a wide area of the hypothalamus and is controlled by a non-ribosomal enzyme, TRH-synthetase [31]. It is stored in the median eminence and secreted when required into the hypophysial portal vessels [32]. TRH binds to membrane receptors on the pituitary cells [33] and increases both the synthesis [34] and the release [35] of TSH. Since TRH has been shown to activate adenyl cyclase [36], this action is believed to be mediated via 3′,5′-cyclic adenosine monophosphate (cyclic AMP). The half-life of TRH *in vivo* is approximately 4 min. It is destroyed in the blood by enzymatic (TRH-degrading enzyme) cleavage of the amide group [37] and excreted via the kidney [32]. The TRH-degrading enzyme is present both in peripheral and hypophysial portal blood of rats but TRH is more rapidly destroyed in the peripheral blood. On the basis of these results, it has been suggested that the portal blood either contains only a low concentration of enzyme or that it contains high concentrations of unidentified substances which act as competitive inhibitors of or substrates for the enzyme [38].

A few active analogues of TRH have been described. It appears that functional groups in the position of imidazole ring are important for the retention of biological activity of the molecule. (Pyro) Glu-pyrazolyl-Ala-Pro (NH_2) is 1½ times as potent as natural TRH when injected into mice [39] but, unlike the gonadotrophin releasing hormone, no very strikingly active analogues with potential therapeutic value have yet been described.

Central noradrenergic, dopaminergic, 5-hydroxytryptaminergic systems have been implicated in the control of TRH production. Woolf, Lee and Schalch [40] found that drugs influencing noradrenergic pathways were ineffective in evoking TSH release and Tuomisto, Ranta, Männistö, Saarinen and Leppäluoto [41] showed that noradrenaline administration decreases serum TSH concentrations, but most of the evidence in the literature indicates that the catecholamine is stimulatory in the control of TRH production. Grimm and Reichlin [42] demonstrated that noradrenaline increases the synthesis of TRH by hypothalamic tissue *in vitro* while Bennett and Edwardson [43] found it evoked the release of the hormone from synaptosome preparations. The centrally acting α-adrenoceptor agonist, clonidine, stimulates the release of TSH [44,45] while α-methylparatyrosine (a drug which depletes brain catecholamines) and phenoxybenzamine, but not propranolol, reduce TSH concentrations. On the other hand,

the catecholamine precursor, L-DOPA, reduces pituitary thyrotrophic activity both in man [46,47] and in rats [44,48]. Moreover, the release of TSH *in vivo* is inhibited by apomorphine, a dopamine receptor agonist [44], suggesting that dopamine, unlike noradrenaline, exerts an inhibitory influence on the functional activity of the hypothalamo-pituitary-thyroid system. A similar inhibitory role has been attributed to 5-hydroxytryptamine(5-HT). Deseril, a 5-HT antagonist, enhances TSH release [49] while 5-HT itself inhibits thyroid activity [50]. Furthermore the indolamine inhibits the release of TRH from hypothalamic tissue *in vitro* when added to the incubation medium [42] and reduces hypothalamic TRH content when implanted in the lateral ventricle or median eminence of rats [51], indicating that 5-hydroxytryptaminergic neurones may inhibit both the synthesis and release of TRH.

In addition to influences from higher centres of the brain, negative feedback mechanisms also play an important part in the regulation of hypothalamo-pituitary-thyroid function. The circulating levels of the thyroid hormones (thyroxine and triiodothyronine) and TSH may influence the production of TRH either by a direct action on the hypothalamus or by affecting higher centres in the brain. Thyroid hormones may also influence the sensitivity of the anterior pituitary gland to the releasing hormone. Recently it has been suggested that TRH may also inhibit its own production by an 'ultra-short loop' feedback mechanism but the physiological significance of this observation is not clear.

GONADOTROPHIN-RELEASING HORMONE (GnRH)

The realization that hypothalamic extracts stimulate the secretion of both FSH and LH from pituitary tissue *in vivo* [52–54] and *in vitro* [55,56] naturally stimulated an intense search for the active substances involved. A hypothalamic fraction with marked gonadotrophin releasing activity was eluted from Sephadex G-25 columns just before vasopressin, suggesting its molecular weight is in the region of 1200–1400 [57,58]. The peptide nature of the hypothalamic hormone was readily demonstrated by its rapid inactivation by peptic or tryptic digestion [59,60]. However, despite this early progress it was not until 1971 that Schally, Arimura, Baba, Nair, Matsuo, Redding and Debeljuk [61] successfully isolated from porcine hypothalami a polypeptide that stimulated the release of both FSH and LH from pituitary tissue. The isolation was achieved by 2 different methods. The first method [61] involved: (1) gel filtration, (2) phenol extraction, (3) chromatography on carboxymethylcellulose columns, (4) rechromatography, (5) free flow electrophoresis, (6) counter current distribution, (7) partition chromatography, (8) column partition chromatography and (9) high voltage zone electrophoresis. The amino acid composition of this GnRH as determined

on acid hydrolyzates was: His 1, Arg 1, Ser 1, Glu 1, Pro 1, Gly 2, Leu 1, Tyr 1 and Trp 1 [61,62]. The second method which led to the isolation of GnRH was simpler and more elegant, being based mainly on counter current distribution [63,64]. The steps in this procedure were as follows: (1) gel filtration, (2) phenol extraction, (3) counter current distribution, (4) chromatography on carboxymethylcellulose columns (5 and 6) counter current distribution. Thin layer chromatography and electrophoresis showed that the material obtained was homogeneous and amino acid analysis confirmed the previous finding of a decapeptide.

The amino acid, sequence (*Figure 4.3*) of porcine GnRH was determined by Matsuo, Baba, Nair, Arimura and Schally [62] using the combined Edman-dansyl procedure [65] coupled with the selective tritation method for C-terminal analysis. These procedures were used directly after enzymatic digestion of the hormone without separation of the fragments. High resolution mass spectral fragmentation of GnRH provided additional data. The proposed structure of porcine GnRH was reinvestigated [66] by Edman-dansyl degradation of the N-terminal pyroglutamyl residue and the amino acid sequence His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly was clearly confirmed. Other experiments revealed that a C-terminal fragment from the chymotryptic digest of GnRH is identical with synthetic Gly-Leu-Arg-Pro-Gly-NH_2 [66], thus confirming the structure provisionally proposed. The structure of porcine GnRH was subsequently reconfirmed by mass spectroscopy [67].

Gonadotrophin releasing hormone was successfully synthesized [62] using a solid phase method. Other successful syntheses of the hormone have since been

(Pyro)Glu–His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–Gly–NH_2	Natural Gonadotrophin-Releasing Hormone
(Pyro)Glu–His–Trp–Ser–Tyr–*D*-Ala–Leu–Arg–Pro–NH–CH_2CH_3	Superactive Gonadotrophin-Releasing Hormone Analogues (From Coy, Coy, Schally, Vilchez-Martinez, Hirotsu and Arimura [88]; Ling, Rivier, Monahan and Vale [90]
(Pyro)Glu–His–Trp–Ser–Tyr–*D*-Lys–Leu–Arg–Pro–NH–CH_2CH_3	
(Pyro)Glu–Trp–Ser–Tyr–*D*-Ala–Leu–Arg–Pro–Gly–NH_2	Analogues of Gonadotrophin-Releasing Hormone which inhibit the action of the natural hormone (From Monahan, Amoss, Anderson and Vale, [93]; Labrie, Savary, Coy, Coy and Schally [94])
(Pyro)Glu–Trp–Ser–Tyr–*D*-Leu–Leu–Arg–Pro–Gly–NH_2	

Figure 4.3. Structures of gonadotrophin releasing hormone and some synthetic analogues

reported using both solid phase techniques [68,69] and classical methods of peptide synthesis [70]. Natural and synthetic GnRH possess identical biological activity [71] and reports to date indicate the peptide has no phylogenetic specificity.

GnRH acts directly on the adenohypophysis to stimulate the synthesis and release of both gonadotrophins [72–75]. It was originally postulated that separate hypothalamic hormones control the secretion of LH and FSH. However, attempts to isolate two such fractions from hypothalamic tissue have failed [76]. Moreover, since antibodies to GnRH block, and analogues of GnRH stimulate, the release of both gonadotrophins, it is now believed that a single hypothalamic regulatory hormone is involved. The differential effects sometimes observed of GnRH on the secretion of LH and FSH are probably the result of changes in pituitary responsiveness induced by the gonadal steroids. The action of GnRH on the adenohypophysis is mediated via the adenylcyclase system [77]. The hormone is subsequently degraded in the blood by enzymatic cleavage of the pyro-Glu-His group and excreted via the kidney [78]. Many analogues of GnRH with varying degrees of activity have now been described (*Figure 4.3*). It appears that pyroglutamic acid, histidine and tryptophan essentially influence the biological activity of the molecule [79]. 1-D-pyroglutamyl-GnRH and 2-D-histidyl-GnRH possess only 8% and 10%, respectively, of the activity of the natural hormone [80]. The gonadotrophin-releasing activity of the molecule is enhanced by substitution of glycine in position 6 with D-leucine, D-alanine or D-tryptophan but the L-isomers are less effective in this respect [81–83]. The increased activity of D-Ala^6-GnRH was originally attributed to a conformation involving a β-11 type of bend in the sequence -Ser-Tyr-D-Ala-Leu- which was thought to be favoured by the hormone receptor [84]. However, more recent studies [85] using (D-Ala^6, (N^{α}-Me)Leu^7)GnRH and ((N^{α}-Me)Leu^7)GnRH tend to rule out this hypothesis. A more plausible explanation of the prolonged gonadotrophin secretion which these analogues cause is that the D-amino acid substitution increases the peptides' resistance to enzymic degradation and thus prolongs its bio-half life [86]. This of course may also explain the oral effectiveness of many of these analogues. Compounds containing the (de-Gly^{10}, Pro^9-NH_2Et) modification [87] in combination with the D-amino acid substitution in position 6 have also been prepared [81,88–90]. These peptides are still more active than their respective decapeptide analogues and some, for example (D-Ala^6, de-Gly^{10}, Pro^9-NH_2Et)GnRH, (D-Leu^6, de-Gly^{10}, Pro^9-NH_2Et)GnRH and (D-Trp^6, de-Gly^{10}, Pro^9-NH_2Et)GnRH are at least 5000% more potent than the natural hormone [90]. The bioactivity of GnRH and its analogues may be prolonged by the formation of hormone conjugates. The biological activity of such conjugates depends on the physicochemical characteristics of the carrier as well as the point of conjugation between the ligand and the carrier. All groups essential

for receptor activation should be left free. Amoss, Monahan and Verlander [91] first described the bioactivity of a GnRH conjugate using (D-Lys^6-GnRH)polyglutamic acid. More recent studies [92] indicate that conjugation of the highly active nonapeptide analogues with polyethyleneglycol, but not with bovine serum albumin, markedly prolongs the duration of action of the hormone. Clearly the development of such compounds with high biological activity and low antigenicity may be of tremendous therapeutic value.

In addition to superactive GnRH analogues, various analogues which inhibit the release of both LH and FSH have also been synthesized (*Figure 4.3*) [90,93–95]. The possibility that such compounds may provide a new method of contraception has naturally stimulated considerable interest in this aspect of the chemistry of GnRH and many analogues of the neurohormone are now being studied. The useful peptides in this respect will be those which (a) have a high affinity for the GnRH receptor but are void of any agonistic activity, (b) are resistant to *in vivo* degradation, and (c) are effective orally.

On the basis of experiments both *in vivo* and *in vitro*, it has been suggested that the catecholamines, noradrenaline and dopamine, may act as synaptic transmitters controlling the secretion of GnRH [96–99]. Most of the evidence available confirms the observation that noradrenaline facilitates the release of GnRH [100–104] and that this effect is mediated via α-adrenoceptors [105] but the possible involvement of a dopaminergic system has become the subject of controversy [106,107]. Schneider and McCann [96] and Kamberi, Schneider and McCann [98,108] claimed that in low doses dopamine stimulates pituitary gonadotrophic activity. However, Sawyer's group [102,109] found that unlike noradrenaline, dopamine administered intraventricularly does not enhance ovulation and other workers have suggested that dopamine may *inhibit* the release of GnRH [100,102–104,110,111].

The role of 5-HT in the control of GnRH secretion is also not fully understood. 5-Hydroxytryptamine, either implanted in the hypothalamus [112] or injected intraventricularly [97,98,108] inhibits gonadotrophin secretion while pinealectomy [113] or administration of parachlorophenylalanine [114,115] stimulates the release of LH and FSH respectively. On the other hand, Collu [116] showed that depletion of brain 5-HT levels with 5,6-dihydroxytryptamine inhibits the secretion of FSH in perpuberal rats. Other reports have been published indicating that 5-HT may play an excitatory role [117,118] and recently it has been suggested that the amine exerts a permissive role in the control of FSH secretion, too high or too low levels being inhibitory [116]. Other indolamines, notably melatonin and 5-methyltryptophol, suppress gonadotrophin secretion *in vivo* [119]. Recent evidence suggests that these two methoxyindoles may act directly on anterior pituitary tissue from neonates to inhibit GnRH-stimulated LH release [120] but the physiological significance of these observations is not understood.

Cholinomimetic drugs and their antagonists have been reported to inhibit and to stimulate respectively gonadotrophin secretion by acting on nicotinic receptors [104,121,122]. However, implantation of atropine into the hypothalamus inhibits the ovarian hypertrophy which normally follows unilateral ovariectomy and induces ovarian atrophy in intact rats [123]. Atropine, injected subcutaneously or intraventricularly also blocks the pre-ovalutory surge of gonadotrophin and prevents the elevation of plasma LH and FSH which normally follows castration [122,124]. Furthermore, intraventricular administration of acetylcholine stimulates the release of LH and FSH, this response is antagonized by atropine and potentiated by the anticholinesterase, neostigmine [125], suggesting that acetylcholine *stimulates* the secretion of GnRH via muscarinic receptors. Such a hypothesis is supported by the observation that acetylcholine facilitates the secretion of GnRH from hypothalamic tissue *in vitro* and that the action is antagonized by atropine [125]. Clearly much further work is required to establish the respective roles of each of these putative neurotransmitter substances in the control of the secretion of GnRH.

Both negative and positive feedback mechanisms also play an important part in the control of hypothalamo-pituitary gonadotrophic activity. Very small doses of oestrogen can inhibit the release of both gonadotrophins [126–128] and larger doses or chronic administration of oestrogen are even more effective in this respect [129]. The inhibitory effects of progesterone are less spectacular than those of oestrogens. Rothchild [130] suggested that progesterone suppresses the ovulatory surge of LH but not the basal secretion of LH. In ovariectamized rats, progesterone, even in very large doses, is a poor inhibitor of LH release [131,132] but, given together with oestrogen, progesterone readily suppresses gonadotrophin secretion [132,133]. Testosterone also inhibits gonadotrophin production but, like progesterone, it is less effective than oestrogen [134]. The sites of the negative feedback action are the subject of controversy. Centres sensitive to testosterone are believed to be located both in the hypothalamus [135] and the pituitary gland [136]. Similarly, centres sensitive to progesterone are present principally in the hypothalamus [137,138] although some experiments in rats and rabbits suggest that progesterone exerts some of its inhibitory effects directly on the pituitary gland [139,140]. Most of the evidence from experiments *in vivo* suggests that oestrogens act on the hypothalamus [141–144] or higher centres of the brain [145] to modulate GnRH production. However, the observation that oestrogens inhibit GnRH-stimulated gonadotrophin release by pituitary segments *in vitro* [146] indicates that a part of the negative feedback action of oestrogen is exerted directly on the pituitary gland. At certain stages of the female cycle, oestrogen and progesterone facilitate the pituitary gonadotrophic response to GnRH. In the rat, the peak plasma oestradiol concentration occurs on the morning of pro-oestrus, before the

ovulatory LH surge [147]. Administration of low doses of oestrogen to immature female rats advances the onset of puberty and stimulates the release of LH from the anterior pituitary gland [148]. Injection of progesterone to a 5-day cyclic rat on the third day of dioestrus advances ovulation by 24 h [149] while administration of the steroid during pro-oestrus increases plasma LH concentrations [150]. These actions of progesterone are dependent on previous exposure of the hypothalamo-pituitary complex to oestrogen [151]. The facilitating effects of oestrogen and progesterone are thought to be the result of direct actions of the steroid on the hypothalamus [145,152,153] and the anterior pituitary gland [153–155] although progesterone, for its full effect, may also require the integrity of connections of the hypothalamus with other areas of the brain.

GROWTH HORMONE-RELEASING FACTOR (GHRF) AND GROWTH HORMONE RELEASE-INHIBITING HORMONE (GHRIH)

Although Bogdanove and Lipner [154] suggested that the hypothalamus may be involved in the control of pituitary somatotrophic activity, some 10 yr elapsed before direct evidence for the hypothalamic regulation of growth hormone secretion was provided. Franz, Haselbach and Libert [155] were the first to describe the somatotrophin-releasing activity of extracts of porcine hypothalami. Their findings were rapidly confirmed by the demonstration that pituitary-stalk median eminence extracts from pigs or cattle stimulate the release of growth hormone from pituitary tissue *in vitro* [156] and that rat hypothalamic extracts evoke the release of growth hormone both *in vitro* [157] and *in vivo* [158]. Studies concerning the hypothalamic growth hormone-releasing factor (GHRH) have involved both bioassay [156,158] and immunoassay [159] of pituitary growth hormone content and release, but some discrepancies between the results obtained with the 2 methods have arisen [160]. Rodger, Beck, Burgus and Guillemin [161] criticized the 'inconsistent results' obtained with the bioassay techniques and their lack of correlation with results from immunoassay. On the other hand, Tulane University workers [162] found studies in which the bioassay only was used quite comprehensible. This group found that the GHRF activity of porcine hypothalamic extracts was abolished by trypsin, chymotrypsin or pepsin and suggested, after preliminary purification procedures, that GHRF may be an acidic polypeptide with a molecular weight of about 2500. Their material was potent and only nanogram quantities were required to evoke both the synthesis (which was blocked by actinomycin D) and the release of GH. Furthermore its activity was not blocked by pretreatment of the assay animals with reserpine, chlorpromazine, sodium pentobarbitone and

Val–His–Leu–Ser–Ala–Glu–Glu–Lys–Glu–Ala	(From Schally, Baba, Nair and Bennett [163])
(Pyro)Glu–Ser–Gly(NH_2)	(From Yudaev, Utesheva, Novikova, Shvachkin and Smirnova [166])

Figure 4.4. Proposed structures of growth hormone-releasing factor

morphine or dexamethasone indicating the hormone acts directly as the pituitary gland. Following this work, Schally, Baba, Nair and Bennett [163] isolated from hog hypothalami a decapeptide (*Figure 4.4*) which they suggested may be the active hypothalamic factor. However, their later studies contradicted this finding [164,165]. A second peptide (*Figure 4.4*) isolated from bovine hypothalamic tissue was shown to stimulate the release of GH from pituitary tissue *in vitro* [166]. This was a tripeptide which was subsequently synthesized by a solid phase method. It was established that the natural and synthetic preparations possessed the same RF value in gel filtration on a Sephadex G-10 column and the same amino acid composition with a blocked N-terminal amino acid. The natural and synthetic peptides were shown to possess identical biological activity but synthetic analogues of the tripeptide in which the C-terminal amino acid was replaced with asparaginamide or the N-terminal amino acid was unblocked (glutamylseryl-glycinamide) were not active. Other workers have, however, been unable to confirm the GHRF activity of the Russian group's tripeptide. The Tulane University workers found it to be ineffective when infused into the hypophysial portal vessels of rats or sheep [167] and when administered intravenously in man [168]. Thus the fine chemical nature of GHRF remains to be elucidated.

While searching for the hypothalamic GHRF, Krulich, Dhariwal and McCann [169] presented data indicating that an inhibitor of GH secretion is also present in hypothalamic fractions from sheep or rats obtained by gel filtration on Sephadex. The fractions inhibit the release of bioassayable GH from pituitary segments *in vitro* [169] and decrease the resting secretion of immunoreactive growth hormone from monolayer cultures of rat pituitary cells [170]. The active substance, which is called the growth hormone release inhibiting hormone (GHRIH) or 'somatostain', was successfully isolated from ovine hypothalamic extracts by means of ion-exchange chromatography, gel filtration, liquid partition chromatography, thin layer chromatography and electrophoresis [171]. The peptide nature of the material was readily demonstrated by its positive Ninhydrin reaction and rapid digestion with chymotrypsin. After quantitative amino acid analysis the sequence of the tetradecapeptide (*Figure 4.5*) was determined by stepwise Edman degradation performed on the intact carboxymethylated peptide, as well as on the unresolved products of tryptic and chymotryptic

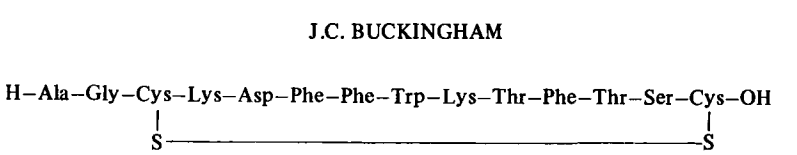

Figure 4.5. Growth hormone release-inhibiting hormone

digests of the peptide. Natural and synthetic preparations were shown to possess identical biological activity [172]. Coy, Coy, Arimura and Schally [173] described the solid phase synthesis of cyclic GHRIH. These workers also showed that a high weight molecular compound formed during the cyclization reaction, believed to be predominantly a dimer, possessed considerable inhibitory activity. Synthetic GHRIH, in either linear or cyclized form, suppresses growth hormone secretion from pituitary tissue both *in vitro* [174] and *in vivo,* in experimental animals [175] and in man [176–178]. It appears to lack phylogenetic specificity.

Using either bioassay methods or the more sensitive immunohisto-chemical techniques, GHRIH has been described in the ventromedial and arcuate nuclei of the hypothalamus, in the tanycytes, in secretory granules of nerve endings of the external zone of the median eminence and in other regions of the brain [179–182]. GHRIH is also present in the rat stomach and pancreas in concentrations similar to those found in the hypothalamus and, in smaller amounts, in the duodenum and jejunum. It has been suggested that the hormone may be involved in local mechanisms controlling pancreatic and gastric secretion [183]. Certainly GHRIH inhibits the secretion of insulin by a direct action on the cells of the pancreas [184] and inhibits glucagon release both in man [185] and in the baboon [186]. In pharmacological doses, GHRIH may also influence the secretion of prolactin, TSH and ACTH but the primary physiological action of the hormone is believed to be the direct inhibition of the somatotrophic activity of anterior pituitary tissue. This action involves 3′,5′-cyclic AMP but the precise site of action of the hormone remains the subject of controversy. French workers [187] suggested that GHRIH is an inhibitor of adenyl cyclase. Such a hypothesis is in accord with the findings that basal and protaglandin (PGE_2)-stimulated 3′,5′-cAMP accumulation is inhibited by 'somatostatin' [174]. However, GHRIH also inhibits theophylline-induced 3′,5′-cAMP accumulation, suggesting it may act at a point following 3′,5′-cAMP formation. Furthermore GHRIH inhibits GH release induced by N^6-monobutyryl-cAMP. The activity of GHRIH is unaffected by inhibitors of protein synthesis and, accordingly, its action is believed to be independent of new protein formation. GHRIH is rapidly metabolised and its half-life *in vivo* is approximately 4 min [176]. It is not, therefore, surprising that single injections of the hormone have little effect on circulating GH levels [188] and that the fall in serum GH induced by continuous infusion of GHRIH persists for only 30 min after the end of the infusion. The

action may be prolonged for a few hours by administration of the hormone as a protamine-zinc complex but, clearly there is a need for superactive analogues which are resistant to biological degradation. Various analogues have been described [189–192]. It appears that deletion of the N-terminal dipeptide (Ala^1-Gly^2) is compatible with high biological activity in rats. With the exception of Ala^2-GHRIH and Ala^5-GHRIH, alanine substituted analogues are less potent than the natural hormone in inhibiting the release of growth hormone from pituitary tissue *in vitro*. On the other hand, D-Trp^8-GHRIH is considerably more effective in this respect [192]. Acetylation of the third residue (Cys^3) renders the molecule less soluble in 0.9% saline and prolongs its action. However, although Brazeau, Vale, Rivier and Guillemin [189] found in rats that a single subcutaneous injection of either *N*-acetyl-Cys^3-GHRIH or *N*-benzoyl-Cys^3-GHRIH inhibits for 24–72 h the secretion of growth hormone normally stimulated by pentobarbitone, their duration of action in man appears to be no longer than that of the natural hormone [190]. Furthermore, unlike pure 'somatostatin', these 'long acting' analogues may induce undesirable side-effects including abdominal pain, vomiting, diarrohea, haemorrhage and thrombocytopenia [190,193].

Central noradrenergic, dopaminergic and 5-hydroxytryptaminergic pathways have all been implicated in the control of pituitary somatotrophic activity. However, because of the inadequate methods available, it is not yet clear whether these pathways influence the secretion of GHRF, GHRIH or both. Artifically-induced rises in brain 5-HT, either by oral administration of the precursor, 5-hydroxytryptophan [194], or by implantation of the indolamine into the lateral ventricle [195] stimulate GH secretion. Moreover, pituitary somatotrophic activity is reduced by treatment with the 5-HT antagonists, cyproheptadine or D-lysergic acid diethylamide, [196,197] or by depletion of brain 5-HT levels with either 5,6-dihydroxytrytamine [193] or parachloraphenylalanine [197]. Accordingly it has been postulated that a 5-hydroxytryptaminergic system stimulates GH secretion. The role of the catecholamines is less well understood. Collu, Fraschini, Visconti and Martini [195] showed that infusion of dopamine, but not noradrenaline, into the lateral ventricle of rats decreases the plasma GH concentration. A similar reduction in circulating GH has been described in man following oral administration of L-DOPA [199]. However, in contrast to these findings, Brown, Krieger, Van Woert and Ambani [200] reported that the dopamine receptor agonist, apomorphine, increases circulating GH in man when given subcutaneously. Ganong [201], using dogs, described a transient rise in plasma GH concentration followed by a fall to the control value during prolonged infusion of L-DOPA. Since the L-DOPA-induced rise in plasma GH concentration is inhibited by the implantation into the third ventricle of an α-adrenoceptor antagonist (phentolamine or phenoxybenzomine) but not a β-

adrenoceptor antagonist (L-propranolol), Ganong [201] proposed that the effect of L-DOPA is mediated via a central α-nordrenergic system. Such a hypothesis is supported by the observation that apormorphine implanted into the third ventricle fails to influence GH levels. The findings of Ganong [201] do not however explain why bromocryptine reduces pituitary somatotrophic activity in acromegalic patients. Clearly, much further work is required to elucidate the true role of the catecholamines.

PROLACTIN RELEASE-INHIBITING FACTOR (PIF) AND PROLACTIN-RELEASING FACTOR (PRF)

The observations that transplantation of the pituitary gland from its normal site to the kidney capsule stimulates mammary growth [202,203] and that sectioning of the pituitary stalk in laboratory animals [204] and in man [205] increases the serum prolactin concentration provide strong evidence for the existence of a prolactin release-inhibiting factor (PIF). Furthermore crude hypothalamic extracts from a variety of species inhibit the release of prolactin from pituitary tissue both *in vivo* and *in vitro* [206–208] although cortical extracts are ineffective in this respect. The chemical nature of the active substance has become the subject of controversy. Purified hypothalamic extracts rich in dopamine and noradrenaline inhibit prolactin release when infused into the hypophysial portal vessels. Both catecholamines are capable of reducing prolactin secretion *in vivo* by a direct action on the pituitary gland although dopamine is the more potent [209,210]. Accordingly, it has been postulated that dopamine and to a lesser extent noradrenaline act as physiological PIFs. Such an hypothesis is supported by various pharmacological studies. For example, perphenazine (α- and β-adrenoceptor antagonist), haloperidol (dopamine receptor antagonist) and phentolamine (α-adrenoceptor antagonist) but not propranolol (β-adrenoceptor antagonist) reduce the *in vitro* action of dopamine on prolactin secretion while apomorphine (dopamine receptor agonist) markedly decreases the amount of prolactin released into the incubation medium [211]. The demonstration that catecholamines (in particular dopamine) reduce prolactin secretion in the rat when implanted into the third ventricle but not when infused in physiological concentrations into the portal vessels [212] and that the dopamine receptor antagonist pimozide increases the plasma prolactin concentration when implanted into the median eminence but not the adenohypophysis, casted some doubt on the proposed role of the catecholamines as physiological PIFs. However, the failure of Kamberi, Mical and Porter [212] to inhibit prolactin secretion by infusion of either dopamine or noradrenaline into the portal vessels may well have been due to the instability in salt solution of the catecholamines

themselves. Recently it has been convincingly shown that infusions of either amine, in concentrations lower than those used by this group, in a glucose solution (which protects them from oxidation) directly into the portal vessels significantly reduces prolactin secretion [209]. Furthermore physiological doses (10–100 ng) of noradrenaline or dopamine, inhibit the release of prolactin both *in vivo* and *in vitro* by a direct action on the pituitary gland [213]. Nevertheless, several groups of workers have suggested that a physiological PIF distinct from the catecholamines may also exist [214–216] and that its secretion may be controlled by a hypothalamic dopaminergic system [100,217–219]. PIF activity has been demonstrated in hypothalamic fractions of which the physicochemical characteristics differ markedly from those of catecholamines [220]. By means of amino acid analysis, thin layer chromatography and mass spectroscopy, the active substances has been isolated and identified as γ-aminobutyric acid (GABA) [221]. Natural and synthetic GABA induce a dose-related inhibition of prolactin release from pituitary tissue *in vitro* and, unlike noradrenaline or dopamine, its actions are not influenced by perphenazine. Moreover, synthetic GABA decreases prolactin release from monolayers of cultured rat pituitary cells and reduces serum prolactin concentrations when administered intravenously.

Although the release of prolactin from the adenohypophysis appears to be predominantly controlled by an inhibitory hypothalamic factor, there is a considerable amount of evidence that a prolactin releasing factor (PRF) is also present in the hypothalamus. The existence of such a factor was first postulated by Meites, Talwalker and Nicoll [222] who showed that subcutaneous administration of hypothalamic extracts induces lactation. The chemical nature of PRF is not known although it has been suggested that it is identical with TRH. Synthetic TRH evokes the release of prolactin from pituitary tissue of a variety of species both *in vivo* and *in vitro* [223,224] and is used clinically as a test for pituitary prolactin reserve [225]. However, more recent evidence indicates that TRH may not be the physiological PRF. The prolactin response to TRH is proportional to the thyroid status but it is not modulated by acute changes in the level of thyroid hormones within the physiological range [226]. Moreover a highly purified bovine hypothalamic fraction distinct from TRH has been shown to enhance the release of prolactin but not other hormones from pituitary tissue *in vitro* [227]. The fraction is believed to contain pepetide material as indicated by its reaction with Lowry reagent and by the formation of ninhydrin-positive components after acid hydrolysis. TRH free fractions have also been isolated from porcine hypothalami and shown to stimulate prolactin secretion *in vivo* and *in vitro* [39,228,229] but again their precise chemical nature remains to be described. In a recent study [230], arginine vasotocin, a polypeptide with corticotrophin releasing activity, was shown to possess prolactin-releasing activity but the physiological significance of this observation is not known.

The mechanisms controlling the production of PRF and PIF by the hypothalamus are not well understood. Several neurotransmitter substances have been implicated but whether they exert their effects on the secretion of PIF or PRF or both remains unclear.

It is widely believed that the secretion of PIF (distinct from dopamine) is governed predominantly by a hypothalamic dopaminergic system [231–233]. Some insight into this mechanism was obtained after the characterization of the tubero-infundibular dopaminergic system [234,235] and it was proposed that these neurones stimulate the secretion of PIF at the median eminence level. A variety of experimental evidence supports this hypothesis. Depletion of hypothalamic biogeneic amines elevates pituitary prolactin production [219], while injection of dopamine or apomorphine directly into the third ventricle increases PIF activity in the portal circulation and depresses prolactin secretion [213,232]. A single intraperitoneal injection of L-DOPA elevates hypothalamic PIF activity and evokes the presence of the 'hormone' in the systemic blood of both intact rats and hypophysectomized rats in which the pituitary gland is transplanted to the kidney capsule [236,237]. Moreover, dopamine evokes the release of PIF from ventral hypothalamic tissue *in vivo* [100]. The roles of noradrenaline and 5-HT in the control of the secretion of PIF and PRF are less well understood. Although noradrenaline possesses PIF activity, several reports in the literature indicate that a central noradrenergic system may stimulate prolactin production either by enhancing the secretion of PRF or by inhibiting PIF secretion. Injection of α-methyltyrosine (an inhibitor of catecholamine biosynthesis) causes a rise in serum prolactin concentration which is readily reduced when catecholamine synthesis is reinitiated by treatment with L-DOPA. However, when α-methyltyrosine-treated rats are injected with dihydroxyphenyl-serine (which normalizes the biosynthesis of noradrenaline but not dopamine), a further rise in the already elevated circulating prolactin levels occurs [231]. Furthermore, selective inhibition of noradrenaline synthesis with disulphiram markedly reduces prolactin secretion [238]. On the other hand, Donoso, Bishop, Fawcett, Krulich and McCann [231] failed to alter circulating prolactin levels by selectively blocking noradrenaline biosynthesis with diethyldicarbonate and Quijada, Illner, Krulich and McCann [100] found that noradrenaline did not influence the secretion of PIF from hypothalamic tissue *in vitro*.

Neither Coppola [239] nor Donoso, Bishop, Fawcett, Krulich and McCann [231] found any definite role for 5-HT in the control of prolactin release. However, Kamberi, Mical and Porter [212] observed a rise in serum prolactin after injection of the amine into the third ventricle. The systemic injection of 5-HT does not alter serum prolactin levels in rats, presumably because it fails to cross the blood brain barrier but a single injection of 5-hydroxytryptophan (5-HTP, a precursor of 5-HT) causes a marked rise in plasma prolactin concentration [240].

5-HTP also significantly increases circulating prolactin levels in man when administered orally; this response is readily inhibited by the 5-HT antagonists, methysergide or cyproheptidine [241,242] indicating that a 5-hydroxytryptaminergic system may also be involved in the regulation of the secretion of PIF and/or PRF. Both GABA and glycine stimulate prolactin release when implanted into the third ventricle but not when injected into the anterior pituitary gland [243]. On the basis of these results, it has been postulated that both these amino acids may also play a physiological role in the control of PIF and/or PRF secretion but further evidence is needed to substantiate this hypothesis.

A positive correlation between serum oestrogen levels and pituitary prolactin secretion has been observed both in experimental animals and human subjects. The patterns of circulating oestrogen and prolactin during the early stages of gestation are similar [244] and the rapid clearance of oestrogen post partum is followed immediately by a fall in plasma prolactin concentration [233]. Yen, Ehara and Siler [245] observed that daily treatment of hypogonadal women with high dose of ethinyl-oestradiol (1 $\mu g\ kg^{-1}$) caused a significant rise in serum prolactin and suggested that a positive feedback relationship between oestrogen and prolactin may exist. A variety of evidence supports such a hypothesis. Elevated circulating prolactin levels have been reported in male patients receiving oestrogen therapy for prostatic carcinoma [246]. Moreover, treatment of rats with oestradiol benzoate increases the prolactin concentration both in pituitary tissue and in the blood [247] and such treatment has been used to induce prolactin-producing pituitary tumours [248]. The site of action of the oestrogens is not clear. Studies *in vitro* suggest the steroids may act directly on the anterior pituitary gland to stimulate the synthesis and release of prolactin [249]. However, oestrogens also deplete hypothalamic PIF [238,249,250]. Buckman and Peake [250] reported that perphenazine-stimulated prolactin secretion is augmented by oestrogen treatment and suggested that the steroid may act on the tubero-infundibular dopaminergic system and thus indirectly modify hypothalamic PIF production. It is possible that oestrogens evoke the secretion of PRF from the hypothalamus but, at present, there is little evidence available to support such a hypothesis.

Since prolactin has no target organ-inhibitory feedback system similar to those of TSH, ACTH, LH or FSH, it was proposed that high circulating levels of prolactin may inhibit the release of the hormone by the pituitary gland [251]. Ample experimental confirmation of this hypothesis has appeared in recent years. Either systemic injection or implantation of minute amounts of prolactin into the median eminence significantly reduces prolactin in the adenohypophysis and blood and inhibits mammary development and lactation in the rat. The inhibitory action of prolactin is believed to be exerted at the hypothalamic level since increases in hypothalamic PIF activity [252,253] and in the activity of the

tubero-infundibular dopaminergic system [218] have been observed in the presence of high circulating prolactin levels. However, again the possibility that production of PRF may also be influenced should not be ignored.

CORTICOTROPHIN-RELEASING FACTOR (CRF)

Corticotrophin-releasing factor (CRF) was among the first of the hypothalamic hypophysiotrophic factors to have its existence postulated but, despite many studies, the precise chemical nature of the 'hormone' remains unknown. The corticotrophin-releasing activity of hypothalamic tissue was first demonstrated by Slusher and Roberts [254]. These workers successfully isolated from bovine posterior hypothalami a lipoprotein which caused adrenal ascorbic acid depletion in intact but not hypophysectomized rats. However, later work indicated that this effect was non-specific and the substance was inactive in rats in which the mobilization of endogenous CRF was prevented either by hypothalamic lesions or by pretreatment with chlorpromazine, morphine or cortisol [255]. Moreover, some doubt concerning the ability of hypothalamic tissue to evoke ACTH release arose and Saffran and Schally [256] showed that the amount of ACTH released *in vitro* by pituitary tissue alone was equal to that released by tissue incubated in the presence of hypothalamic, neurohypophysial or liver tissue. The first direct evidence of the existence of a hypothalamic CRF came from the experiments of Porter, Vanatta and Dillon [257]. It was shown that extracts of plasma from the sella turcica of stressed hypophysectomized dogs caused adrenal ascorbic acid depletion in cortisol-treated rats. No similar activity was found in the carotid blood of the same dogs and it was suggested that the corticotrophin-releasing activity of the portal blood was due to a substance acquired by the blood on its passage through the primary capillary plexus of the hypophysial portal vessels [258,259]. Later studies confirmed and extended these observations [260,261]. The ability of crude hypothalamic extracts to stimulate the secretion of corticotrophin (ACTH) *in vivo* in rats with hypothalamic lesions [262–264] or in animals in which the release of CRF was inhibited by corticosteroids [260] was subsequently described. Moreover, in contrast to the earlier findings, it was convincingly demonstrated that hypothalamic extracts, but not cortical extracts, evoke the release of ACTH from pituitary tissue *in vitro* [265], thus providing firm evidence for the existence of a hypothalamic CRF.

Although all attempts to isolate and identify CRF so far have failed, it is widely believed that the 'hormone' is a small polypeptide. After preliminary purification procedures, CRF activity is evident in a plasma protein fraction of portal blood. Moreover, the CRF activity of hypothalamic extracts is readily

inactivated by the proteolytic enzymes, pepsin or trypsin. For some time it was thought that vasopressin may be chemically identical with CRF [266–268]. Certainly small doses of vasopressin evoke the release of ACTH from pituitary tissue *in vitro* [269], increase adrenocortical activity in normal but not in hypophysectomized rats [270], and stimulate pituitary adrenocorticotrophic activity in rats in which endogenous CRF release is prevented by pretreatment with pentobarbitone and chlorpromazine. Furthermore, immunofluorescent studies have shown a rise in the vasopressin content of the median eminence of adrenalectomized rats [271] which can be prevented by dexamethasone treatment [272]. However, these results should be interpreted with some caution since the antibody may cross-react with other putative CRFs, notably arginine vasotocin. A great deal of circumstantial evidence supports the hypothesis that vasopressin is CRF. McCann and Brobeck [266] showed that median eminence-lesioned rats develop diabetes insipidus and that ACTH release can be elicited in such animals by injection of vasopressin. Moreover, there is an association between the intensity of the diabetes insipidus and the degree of inhibition of pituitary adrenocorticotrophic activity in animals with hypothalamic lesions [273] and the ascorbic acid depletion in such lesioned rats treated with vasopressin is proportional to the pressor activity. Stress often results in the release of both vasopressin and ACTH [274]. The primary capillary plexus in the median eminence is in close contact with the neurones of the supra-optico hypophysial tract. Neurosecretory substances do enter the hypophysial portal vessels and recent evidence [275] suggests that vasopressin may be secreted directly into the portal vessels. Although there can be no doubt that vasopressin possesses corticotrophin releasing activity, a considerable amount of evidence suggests that it is not the only CRF and that it is probably not the major hypothalamic factor that triggers ACTH release. According to Saffran and Saffran [276], the doses of vasopressin used by McCann and Fruit [273] to induce ACTH release in hypothalamic-lesioned rats is several thousand times greater than the dose needed to inhibit diuresis maximally. This criticism is however not necessarily valid as there is no data available to indicate how much systemic vasopressin is necessary to change the concentration in the hypophysial portal vessels [277]. De Wied, Smelik, Moll and Bouman [262] showed a great deal of CRF activity, but little pressor activity, in extracts of hypothalami from hypophysectomized rats, while McDonald, Wagner and Weise [278] found a complete lack of correlation between the release of these 2 hormones in response to nicotine, water loading and water deprivation in man. Hypothalamo-pituitary-adrenocortical activity *is* reduced in rats with hereditary diabetes insipidus but hypothalamic extracts from such rats *possess* corticotrophin-releasing activity (Buckingham and Leach, unpublished observations) and the animals are capable of responding to stress with a rise in plasma corticosterone concentration [279]. Both hypothalamic

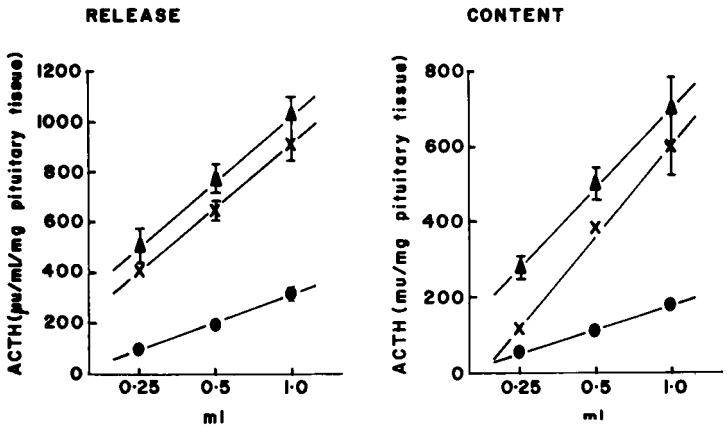

Figure 4.6. Effects of hypothalamic extracts, lysine vasopressin and arginine vasotocin on the adrenocorticotrophic activity of pituitary tissue in vitro. X———X, *hypothalamic extract (1.0 ml contains the equivalent of 1 hypothalamus);* ●———●, *lysine vasopressin (100 mu* ml^{-1}*);* ▲———▲, *arginine vasotocin (1* $\mu g\ ml^{-1}$*). Each point is the mean of 5 determinations shown with its standard error. Where standard errors are omitted they were within 5% of the mean value [269,280]*

extracts and vasopressin stimulate the synthesis and release of ACTH by pituitary segments *in vitro* (*Figure 4.6*). However, the deviation from parallelism of the dose–response lines of hypothalamic extracts and vasopressin is very highly significant, indicating that the corticotrophin-releasing factor in the tissue extract is not chemically identical with vasopressin [269,280]. Similar results have been described using an isolated pituitary cells superfusion system [281]. Furthermore, although CRF and vasopressin are stored in nerve endings in the hypothalamus, they are apparently localized in different synaptosomes suggesting that the production, transport and release of CRF and vasopressin takes place in separate neurones within the hypothalamus [282].

Various attempts have been made to isolate CRF from tissue of both neurohypophysial and hypothalamic origin. (Neurohypophysial tissue also possesses CRF activity). Schally, Saffran and Zimmerman [283], using "protopituitrin" (the starting material for vasopressin isolation) as the source of CRF obtained two fractions with CRF activity which they designated α-CRF and β-CRF. The β-fraction was the more potent and also possessed pressor and anti-diuretic activity. A preliminary investigation of its structure suggested a resemblance to lysine vasopressin (*Figure 4.7*) [284]. α-CRF, which also possessed ACTH-like activity [285] was later described as 2 peptides, α_1-CRF and α_2-CRF, both similar to but slightly larger than α-MSH. On the basis of these findings, it was postulated that α-CRH is merely a precursor of ACTH (the sequence of the first

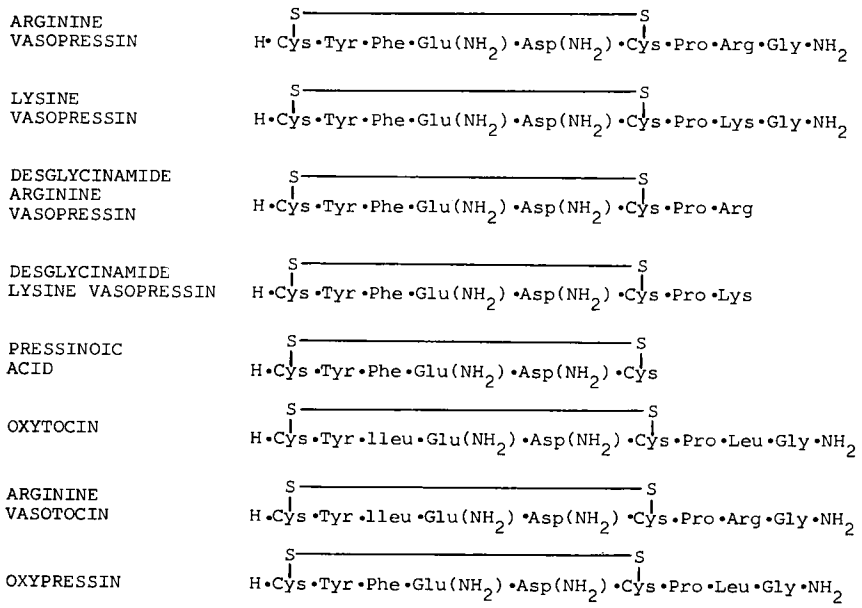

Figure 4.7. Amino acid sequence of vasopressin and related peptides

thirteen amino acids of ACTH is identical with α-MSH) and that β-CRF is the neurohumoral transmitter which stimulates ACTH release [286]. Later the same workers turned to using hypothalamic tissue for their studies which, of course, provides a more physiological source of CRF [287,288]. They found that this yielded 2 fractions with CRF activity which had chromatographic properties similar to those of α- and β-CRF obtained from the posterior pituitary gland. However, in contrast to β-CRF of neurohypophysial origin, the corresponding fraction from the hypothalamus had considerable ACTH-like activity and the others concluded that the fraction that resembled α-CRF was probably the physiological corticotrophin-releasing-hormone.

In another study, a substance with CRF activity was isolated from sheep hypothalami by means of gel filtration and ion-exchange chromatography [289]. The substance, whose structure remains to be determined, has no pressor or ACTH-like activity and is believed to be a single chemical entity. However, the material was not subjected to countercurrent distribution which may separate the 2 CRF activities and thus the possibility of a second CRF cannot be disregarded. Chan, Schaal and Saffran [290] demonstrated two 'CRF's' in rat median eminence tissue, a large and a small CRF. They suggested that the

activity may reside in a peptide containing aromatic amino acids but none of this evidence is unequivocal.

The use of differential bioassay techniques has provided another approach to the determination of the chemical nature of CRF. Since CRF is believed to be a polypeptide similar to vasopressin, valuable information concerning its chemical nature may be obtained by comparing the dose–response relationships of various polypeptides related to vasopressin with those of hypothalamic extracts using two or more bioassay systems for the determination of CRF activity. Many vasopressin analogues (*Figure 4.7*) have now been tested for their ability to release ACTH employing a sensitive and precise *in vitro* assay technique using rat pituitary segments [269] and an *in vivo/in vitro* system using pentobarbitone–chlorpromazine-treated rats [291]. Both methods demonstrated the abilities of arginine- and lysine-vasopressin to produce dose-related increases in corticotrophin production. The desglycinamide derivatives of lysine- and arginine-vasopressin exhibited some activity in the *in vitro* system but were ineffective when injected into the pentobarbitone–chlorpromazine-treated animals. Pressinoic acid, its amide, oxytocin, alanine-8-oxypressin and the 'tail fragment' (proline–arginine–glycinamide) of arginine vasopressin did not increase corticotrophin production using either method. Oxypressin and arginine vasotocin exhibited very marked corticotrophin-releasing activity in both assay systems [292] and, unlike lysine vasopressin, the dose–response relationships of the latter, closely resembled those of hypothalamic extracts (*Figure 4.6*) [269].

There is no evidence that this observation has any physiological significance but, in this respect it is interesting to note that arginine vasotocin has been demonstrated in the pineal gland, cerebrospinal fluid and hypothalamus of certain mammalian species. However, as yet, no physiological role has been assigned to it.

One of the major problems associated with the isolation of small relatively unstable polypeptides from biological tissue is that the peptides may break down or undergo other chemical transformation during the extraction procedures. Recently, a new approach to the study of the chemical nature of CRF has been developed [293] which overcomes this problem. Jones, Hillhouse and Burden [293] demonstrated that 5-hydroxytryptamine selectively stimulates the release of CRF, but not vasopressin, from hypothalamic tissue *in vitro* and attempts are now being made to isolate and identify the 'CRF' in the incubation medium. Preliminary separation by chromatography on Sephadex G-25 demonstrated two peaks of CRF activity, fraction A and B, with molecular weights of around 2500 and 1300 respectively. Both fractions evoked dose-related increases in pituitary ACTH release either when injected into basal hypothalamic-lesioned rats or when incubated with adenohypophysial tissue *in vitro* but Fraction A was the more potent in this respect. Neither fraction markedly influenced the release of

other pituitary hormones. It is hoped that the amino acid sequence of these peptides will soon be determined and thus that the chemical nature of the corticotrophin-releasing factor(s) will finally be elucidated. Of course, this type of method may also be of considerable potential value in the identification of other hypothalamic hormones.

Although the chemical nature of the corticotrophin-releasing hormone is not known, a great deal of attention has focussed on the mechanisms controlling its production. Results from experimental studies both *in vivo* and *in vitro* have suggested that a variety of neurotransmitter substances may be involved. The possibility that a central cholinergic mechanism may control the release of CRF was first proposed by Endröczi, Schrieberg and Lissák [294] who showed that implantation of carbachol into various regions of the central nervous system stimulates pituitary adrenocorticotrophic activity. A considerable amount of evidence now supports this hypothesis. For example, implantation of atropine into the anterior hypothalamus reduces the 17-hydroxycorticosteroid response to stress [295–297] and carbachol increases the plasma corticosterone concentration when infused into the lateral ventricle of rats [298]. Furthermore, pg quantities of acetylcholine stimulate both the synthesis and release of CRF by the isolated rat hypothalamus *in vitro* [299–301]. Since both pilocarpine [302,303] and nicotine [304] stimulate hypothalamo-pituitary adrenocortical activity *in vivo* and atropine and hexamethonium antagonise the stimulatory effect of acetylcholine on hypothalamic CRF production *in vitro* [305, Buckingham and Hodges, unpublished observations], it appears that both nicotinic and muscarinic receptors are involved. The role of 5-hydroxytryptamine in the control of CRH secretion is less well understood. Studies *in vivo*, involving implantation of 5-HT either into the lateral ventricle or into various regions of the hypothalamus suggest that the indolamine has no effect on the basal secretion of CRF but inhibits its release in response to stressful stimuli [306–308]. Several other findings support this theory [309,310] but most of the evidence suggests that a 5-hydroxytryptaminergic system exerts a *stimulatory* influence over the functional activity of the hypothalamo-pituitary-adrenocortical complex. Krieger and Rizzo [311] first proposed that the circadian periodicity of 17-hydroxycorticosteroids is controlled to some extent by 5-HT. More recent studies indicate that the amine is also important in the regulation of stress-induced adrenocortical function. Results from experiments both *in vivo* and *in vitro* substantia these observations. Infusion of 5-HTP, a precursor of 5-HT, into the rhesus monkey or man [313] significantly elevates the plasma cortisol level while cyproheptadine (a 5-HT receptor antagonist) reduces the plasma cortisol concentration both in normal subjects and in patients with Cushing's syndrome of 'hypothalamic origin' [314–316]. Very small doses of 5-HT stimulate both the synthesis and release of CRF by hypo-

thalamic tissue *in vitro* [300,301,317]. Moreover, the effects are completely inhibited by appropriate doses of methysergide, suggesting that 5-HT is acting at specific 5-HT receptors [317]. An early report indicated that vasopressin may facilitate the release of CRF [318] but experiments *in vitro* suggest that vasopressin is ineffective in this respect [319].

Central GABA minergic and adrenergic nervous pathways are believed to exert a tonic inhibitory influence over the hypothalamic secretion of CRF. Both the basal and stress-induced activity of the hypothalamo-pituitary-adrenocortical complex in the rat are reduced when GABA is implanted into the third ventricle but enhanced by the implantation of the GABA receptor antagonists, picrotoxin and bicuculline [320]. Furthermore, small doses of GABA markedly reduce both acetylcholine- and 5-HT-stimulated CRF production by isolated rat hypothalami *in vitro* and the effects are inhibited by picrotoxin [301,317]. The catecholamine precursor, L-DOPA, inhibits the 17-hydroxycorticosteroid response to stress in dogs when administered intravenously [321] or into the third ventricle [322]. Similar inhibitory effects are also evident following implantation of L-noradrenaline, dopamine, α-ethyltryptamine (a monoamine oxidase inhibitor) or tyramine (a drug that releases catecholamines from nerve endings) into the third ventricle [322]. Inhibition of catecholamine biosynthesis by administration of α-methyl-para-tyrosine (an inhibitor of tyrosine hydroxylase) results in a rise in plasma corticosterone [323] and ACTH [324,325] concentrations. On the basis of these and other experimental findings, it was proposed that both noradrenergic and dopaminergic mechanisms are involved but more recent evidence indicates that only the former is of any functional significance. Apomorphine, implanted into the third ventricle of dogs, fails to influence pituitary-adrenocortical activity. Inhibition of dopamine-β-hydroxylase with bis(1-methylhexahydro-1,4-diazepinyl-4-thiocarbonyl)disulphide decreases the hypothalamic noradrenaline content and increases ACTH secretion while administration of dihydroxyphenylserine causes a selective decrease in hypothalamic dopamine and overcomes the rise in ACTH secretion induced by α-methylparatyrosine [201]. Moreover, noradrenaline inhibits both basal and acetylcholine- or 5-HT-stimulated CRF secretion from the rat hypothalamus *in vitro* but dopamine is ineffective in this respect [301,317]. Despite some reports to the contrary, most of the evidence in the literature indicate that the inhibitory influence of noradrenaline is mediated via α-adrenoceptors [305,326–328].

Studies in adrenalectomized, adrenal-enucleated and intact animals have shown that under 'non-stress' conditions an inverse relationship exists between the blood corticotrophin and corticosteroid concentrations, indicating that the blood corticoids play an important role in the regulation of the functional activity of the hypothalamo-pituitary-adrenocortical system under basal non-stress conditions [329–333]. The observations that the stress-induced release of

ACTH is greatly exaggerated in adrenalectomized rats [330,334–336] but inhibited in animals receiving *prolonged* corticosteroid treatment [337–340] led to the conclusions that the blood corticoids are also important in the control of stress-induced hypothalamo-pituitary-adrenocortical activity. However, the results of experiments involving the acute administration of corticoids indicate that this is not the case. Smelik [341] found no direct correlation between the plasma corticosterone concentration and inhibition of stress-induced ACTH release in rats given large doses of the steroid either intraperitoneally or subcutaneously but that inhibition of ACTH release occurred only some time after the blood corticosterone concentration returned to the resting level. This observation has been confirmed using both direct and indirect indices of ACTH secretion [333,342] and the concept of a 'delayed feedback mechanism' controlling the release of ACTH in response to stressful stimuli is now widely accepted. Recently a 'rapid feedback mechanism' has also been implicated in the control of stress-induced hypothalamo-pituitary-adrenocortical activity. Dallman and Yates [343] demonstrated that infusion of corticosterone inhibits ACTH secretion in response to stress but the duration of action of the steroid is very short (<5 min). This observation was confirmed by Jones, Brush and Neame [344] who found the system to be sensitive to the rate of increase of the plasma concentration of corticosterone but to be saturated at concentrations outside the 'physiological range'. However, these experiments were performed under grossly non-physiological conditions which cast some doubt on the significance of the results.

The adenohypophysis, the hypothalmus and higher centres in the brain have all been proposed as possible sites of action of the corticosteroids. Evidence for inhibition at the pituitary level has been obtained from studies both *in vivo* and *in vitro* and it appears that the corticoids act directly on the adenohypophysis to inhibit the release but not the synthesis of ACTH induced by CRF [269,345–348]. Recent studies indicate that the hypothalamus is 'more sensitive' to the inhibitory influences of corticosteroids [349]. Certainly corticosteroids inhibit adrenocortical activity when implanted into the hypothalamus [350,351]. Moreover, very small doses of corticosteroid inhibit the secretion of CRF by isolated hypothalami *in vitro* [301,349]. Corticosteroid treatment reduces the hypothalamic CRF content [352,353] and prevents the release of CRF from already increased stores in the median eminence of adrenalectomized rats [354] and depresses hypothalamic unit activity [355]. However, in these experiments, the corticosteroids were given either orally or systemically and thus the results may be the consequence of the steroid's action either on the hypothalamus or on higher centres in the brain. Substantial evidence exists that corticosteroids may exert feedback effects at extrahypothalamic sites in the central nervous system [356–359]. The possible involvement of the amygdala

and the hippocampus as sites of corticosteroid feedback has been well documented and the possibility that other brain areas (notably the thalamus, basal septal area and the rostral midbrain reticular formation) may also be involved should not be disregarded [360,361]. Clearly much further work is necessary in order to elucidate the relative importance of these sites. From time to time, the possibility has been raised that a short loop feedback mechanism exists, in which ACTH restrains CRF secretion without the interposition of corticosteroids. The results of many experimental studies support the existence of such a mechanism [362,363] but its physiological significance is not understood.

MELANOCYTE-STIMULATING-HORMONE RELEASE-INHIBITING FACTOR (MSH-RIF) AND RELEASING FACTOR (MSH-RF)

The hypothalamus exerts both inhibitory and stimulatory influences on the release of MSH from the pars intermedia [364,365] but some confusion exists as to the identity of the true hypothalamic hormones which control MSH secretion under physiological conditions. Nair, Kastin and Schally [366] successfully isolated and identified a tripeptide with marked MSH-RIF activity from bovine hypothalami. However, although the peptide (H-Pro-Leu-Gly-NH_2) inhibits MSH secretion in frogs and rats [367,368], it is ineffective in man [369,370]. In a recent study [371], it was postulated that mammalian TRH [Pyro-Glu-His-Pro-NH_2] may be a physiological MSH-RF in frogs. These workers showed that TRH and some of its analogues stimulate the release of α-MSH from frog pituitary tissue *in vitro*; the effective analogues were those in which the histidyl residue remained intact. Mammalian TRH is present in the hypothalamus of amphibians [372] but does not appear to influence TSH secretion [30]. No mammalian MSH-RH has yet been identified.

The pathway by which MSH-RIF and MSH-RF reach the pituitary gland is unclear. Unlike the pars distalis, the pars intermedia is well supplied with neurones from the hypothalamus but its blood supply is poor. Accordingly it has been suggested that the hypothalamic hormones are released from nerve endings within the pars intermedia, but further experimental evidence is needed to substantiate such a hypothesis.

LOCALIZATION OF THE HYPOPHYSIOTROPHIC HORMONES

Although the hypothalamus is considered the source of peptide hormones which influence the pituitary gland and other target tissues outside the brain, the specific cells which produce these hormones have yet to be isolated. Because of the extreme complexity of its cellular structure compared with other endocrine organs, the precise site of production of many of the hypothalamic hormones

remains obscure. Assay of even a very small piece of hypothalamus obtained by microdissection enables only the area and not the precise cells producing the hormone to be determined. More detailed information has been obtained from experiments involving the placement of electrolytic lesions in specific hypothalamic regions [373] or by evaluation of the releasing-hormone concentration in the 'hypothalamic islands' of rats submitted to hypothalamic deafferentation [374]. However, most of our knowledge concerning the site of neurohormone production has come from histological studies. The classical work on the magnocellular neurosecretory system of the hypothalamus demonstrated the formation of neurosecretory material in the perikarya of supraoptic and paraventricular neurones and its transportation along the nerve axons to the posterior pituitary gland. However, these techniques are not sensitive enough to detect the hypothalamic-releasing hormones. Recently highly sensitive chemical immunofluorescence methods have been developed in an attempt to localize these hormones. Preliminary studies using these techniques have confirmed directly that oxytocin and vasopressin are secreted by the magnocellular system and indicated strongly that the hypophysiotrophins are synthesized within the parvicellular nuclei. The gonadotrophin-releasing hormone (GnRH) and 'somatostatin' have been demonstrated in granules within axon terminals close to the portal capillaries in the median eminence, suggesting that the releasing hormones are transported along the axons of the parvicellular nuclei and secreted directly into the portal blood vessels. However, direct neurosecretion from the parvicellular structures to the portal vessels may not be the only route by which the releasing-hormones reach their target organs. Hypothalamic hormones have now been demonstrated both in regions of the hypothalamus outside the parvicellular and magnocellular structures and in the cerebrospinal fluid (CF) [275]. The physiological significance of these observations is not known but recent evidence suggests that releasing hormones may also pass directly from the CSF to the portal blood. Injection of thyrotrophin-releasing hormone into the third ventricle causes rapid release of thrytrophin from the pituitary gland while a similar injection of GnRH facilitates the release of the gonadotrophins. Histological examination of the ependymal cells of the median eminence has demonstrated the presence of elongated cells, the tanycytes, interposed between CSF and portal capillaries which are belived to perform a transport function. The cells have a variable number of ciliated microvilli projecting into the third ventricle and a basal process which intermingles with the perivascular basement membrane of the portal capillaries (*Figure 4.8*). The fact that the cells are capable of absorbing endogenous peroxide from the CSF suggests that transport may occur from the CSF towards the basal processes but the possibility that transport may occur in the opposite direction cannot be disregarded. The full physiological role of the tanycytes is not understood. Clearly they may be

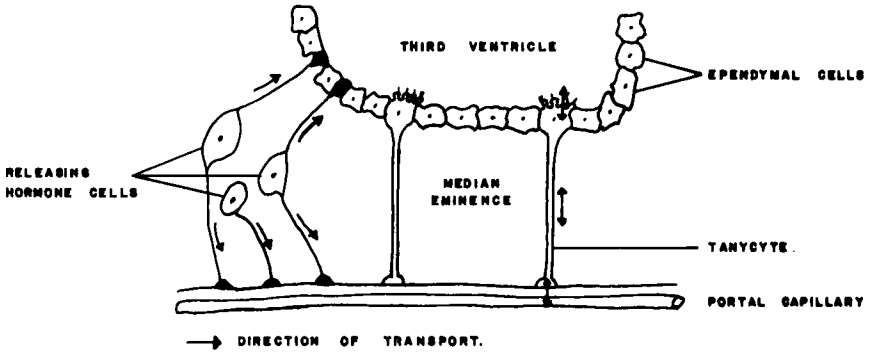

Figure 4.8. Means by which hormones may be transported between the hypophysial portal blood vessels and the cerebrospinal fluid [1a]

associated with the transportation of the releasing hormones and substances which influence their production. However, they may also be concerned with the passage of substances from the systemic circulation to the CNS. Indeed, they may provide an important route by which steroids and pituitary hormones enter the CSF to influence the production of the hypothalamic releasing hormones by negative feed back mechanisms.

CLINICAL USE OF HYPOPHYSIOTROPHIC HORMONES

Until the introduction of synthetic thyrotrophin-releasing hormone (TRH) and gonadotrophin-releasing hormone (GnRH), clinical neuroendocrinology did not exist as a separate discipline. The availability of these hypothalamic regulatory hormones permits the direct evaluation of pituitary function for the first time [375]. Consequently attempts can now be made to study the capacity of the pituitary gland to produce the trophic hormones, thus enabling accurate differentiation between diseases of the pituitary gland and the hypothalamus as apparent causes of pituitary dysfunction. The standard TRH test [376] has certainly proved a useful tool in the diagnosis of thyroid disease and in the assessment of pituitary TSH reserve in patients with pituitary lesions [377]. In this test, the blood TSH concentration is estimated at various times after intravenous administration of TRF. Basal triiodothyronine (T_3) and thyroxine (T_4) are also assessed. In patients with primary hypothyroidism, TRH causes an exaggerated rise in the already elevated TSH levels while those with hypothyroidism due to pituitary disease usually fail to respond. Because of the already high circulating levels of T_3 and T_4, TRH is also generally ineffective in subjects with hyperthyroidism but, with this test, it is not possible to distinguish between hyperthyroidism and, for example, autonomous thyroid adenomata

[378]. Thyrotrophin-releasing hormone also exhibits marked prolactin-releasing activity and, accordingly, it is also used in investigations of abnormal prolactin production [379]. A similar standard GnRH (in which LH and FSH are estimated at various times after intravenous administration of GnRH) is also of considerable diagnostic value [380]. Like the TRH test, it provides a test for pituitary reserve and enables differentiation between brain-hypothalamic and pituitary disease. Since pure corticotrophin releasing hormone is not available, there is no comparable test for use in the diagnosis of pituitary-adrenal dysfunction. Vasopressin has often been used as a test for pituitary ACTH reserve. However, vasopressin is not chemically identical with corticotrophin-releasing hormone [269] and the validity of this test is doubtful.

In addition to their diagnostic usefulness, the hypothalamic-releasing hormones and their superactive analogues are of potential value in the treatment of hypothalamic and pituitary disease. However, the result of treating patients suffering from infertility with GnRH have been disappointing. Increases in sperm count and motility have been reported in patients with oligospermia but the counts later fell, despite continued therapy. Attempts to induce ovulation with GnRH have also been relatively unsuccessful although Besser and Mortimer [375] have reported some success in the treatment of secondary amenorrhea in patients with anorexia nervosa. However, clinical trials are now in progress with many of the superactive analogues of GnRH and the results are rather more promising. Treatment with pharmacological agents known to influence the activity of releasing hormone cells has been effective in the treatment of some endocrine disorders. There are many reports of successful attempts to induce ovulation in patients suffering from infertility by treatment with bromocryptine, a dopamine receptor agonist. Bromocryptine also effectively reduces blood prolactin levels in subjects with hyperprolactinaemia and paradoxically reduces growth hormone production in acromegalic patients, while cyproheptadine, a 5-hydroxytryptamine antagonist, has been successful in the treatment of 'hypothalamic Cushing's syndrome'. Clearly a deeper understanding is necessary of the neural pathways within the hypothalamus which influence the synthesis and release of the hypothalamic hormones so that 'releasing hormone therapy' is superceded, at least to some extent, by treatment with neurotransmitter agents, their agonists and antagonists.

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5 Copper Complexes - A Unique Class of Anti-Arthritic Drugs

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INTRODUCTION

The first report that a copper (Cu) complex was effective in the treatment of rheumatoid and other degenerative connective tissue diseases appeared in 1941. Since the first study of Cu complexes in an animal model of inflammation was not carried out until 1950, their use in human therapy preceded pharmacologic evaluation by about 10 yr. Subsequent demonstrations that Cu complexes have anti-inflammatory activity in many animal models of inflammation provides support for the earlier claims that they were effective in treating rheumatoid and other degenerative connective tissue diseases in man.

In 1938, before Cu complexes were used in therapy, it was reported that patients with rheumatoid arthritis had higher than normal total serum Cu concentrations which returned to normal levels with remission. Had this increase in total serum Cu been recognized as an increase in serum Cu complexes, this increase might have been considered to be a physiologic response to this disease when Cu complexes were subsequently found to have anti-arthritic activity in man and anti-inflammatory activity in animal models of inflammation.

The lack of recognition that Cu complexes were effective therapy as a result

of their ability to mimic the physiologic response to inflammatory disease is likely due, in part, to the lack of an awareness that Cu was required for any biochemical process in normal metabolism. However, the essentiality of Cu or its requirement in at least one mammalian biologic process had been reported in 1928, 10 yr before the observed physiological change in arthritic patients, 13 yr before the use of Cu complexes in arthritic diseases, and 22 yr before the first pharmacological evaluation. Since then, there have been many studies demonstrating its biochemical requirement in human metabolism.

Similarly, following the first demonstration in 1938 that the serum Cu concentration is higher than normal in rheumatoid arthritis, this has been confirmed many times subsequently. Reports of the successful therapeutic use of Cu complexes as anti-arthritic drugs in nearly 1500 patients have appeared in the published literature from 1941 until as recent as 1974. Pharmacological studies demonstrating the anti-inflammatory activity of Cu complexes in many of the recognized animal models of inflammation have been published from 1950 to the present. Unfortunately, all of these biochemical, physiological pharmacological and clinical studies have been ignored because of our lack of knowledge concerning Cu and its requirement for biologic processes.

This lack of knowledge is most likely due to our failure to include the chemical, biochemical, physiological, pharmacological and clinical aspects of essential metal metabolism in our basic science scholastic courses.

It is hoped that this review will stimulate the reader's interest not only in Cu metabolism but in all essential metal metabolism and the interrelationships of the various essential metal dependent processes. For the medicinal chemist, this may suggest many interesting new approaches to the synthesis of more effective drugs where they exist for therapy of some diseases, and new drugs for the therapy of diseases which have resisted successful therapeutic solutions using classical approaches for new drug development.

PHYSIOLOGY OF COPPER COMPLEXES

Copper is one of a number of metals which are known to be 'essential' because they, like essential amino acids, essential fatty acids and essential cofactors, are required for normal metabolism but are not synthesized *de novo*. The essentiality of Cu was established by Hart, Steenbock, Waddell and Elvehjem [1] in 1928 when they demonstrated that it was required for the synthesis of haemoglobin. Since that time, Cu has been shown to be required for growth, keratinization, pigmentation, bone formation, reproduction, fertility, development of the central and peripheral nervous systems, cardiac function, cellular respiration, nerve function, extracellular connective tissue formation, and regulation of

monoamine concentrations [2]. Tissue requirements for Cu are believed to be met and controlled in a homeostatic fashion based upon availability, absorption, storage, utilization and excretion [3]. All normal tissues contain Cu in the form of Cu-dependent components which include metalloproteins, metallo-enzymes and lower molecular weight complexes of biological importance [2].

Copper has received considerable attention with regard to its presence in normal blood, plasma and serum. A survey of this literature [4] revealed that total-serum (TS) Cu concentractions were most commonly determined and reported to be between 94 and 133 μg 100 ml^{-1}. Variation in these values is in part due to geographic location, which may effect dietary intake, but sample contamination and analytical error have been used to account for marked deviations from the currently accepted norm of 106 ± 5 μg 100 ml^{-1} for males and 114 ± 5 μg 100 ml^{-1} for females [2,5]. Larger than usual mean TS Cu concentrations may also be the result of including individuals in a 'normal' population who have an infection or inflammation of one sort or another, since these cause an elevation in TS Cu concentrations [6–14]. Total serum Cu concentrations have also been reported to increase slightly with age in adults [15,16].

Total serum Cu has three principal forms. Ceruloplasmin (Cp) (0.34% Cu), an α_2-globulin with oxidase activity, is synthesized in the liver and increases in serum as an acute phase reactant. It accounts for 80 to 90% of serum Cu [14]. The remaining non-ceruloplasmin Cu is also an acute phase reactant and is released from liver stores as albumin and amino acid complexes which serve in its transportation and utilization [2,17,18]. A portion of this non-ceruloplasmin Cu is contained in transferrin, the metallo-enzyme responsible for transportation and utilization of iron in haemoglobin synthesis.

Sex-related variation in serum Cu has been attributed to observations that oestrogens increase TS Cu values [19–21]. This is supported by 2 recent reports that normal women have significantly higher serum Cu concentrations than do normal males [15,22].

PHYSIOLOGY OF COPPER COMPLEXES IN RHEUMATOID ARTHRITIS

Over the last 40 yr, studies of tissues from individuals with rheumatoid arthritis (RA) have revealed that Cu concentrations change in this disease state. Results reported from 1938 to 1953 are presented in *Table 5.1.* Heilmeyer and Stuwe [6] were first to report a total plasma Cu increase in men and women with active RA which returned to normal with remission. These changes in TS Cu were not related to sex but were most pronounced in active febrile disease and correlated with a decrease in haemoglobin and an increase in erythrocyte sedimentation rate (ESR) [7]. This arthritic disease-related alteration of serum Cu was con-

Table 5.1. REPORTED ALTERATIONS OF COPPER(Cu) CONCENTRATIONS IN RHEUMATOID ARTHRITIS FROM 1938 TO 1952

Sex	*Copper* [a]		*RBC Copper* [a,b]		*Whole Blood Cu* [a]		*References*
	normal	*arthritic*	*normal*	*arthritic*	*normal*	*arthritic*	
?	105 (80–140)	210					[6]
M	106 ± 16 (15)						[5]
F	107 ± 17 (15)						
MF		173 (23) (118–216)					
M	123 (12) (85–152)	179 (2) (140–217)	129 (7) (96–169)	100 (2) (60–140)	129 (8) (90–160)	149 (2) (117–181)	[24]
F	131 (12) (103–152)	192 (6) (110–250)	120 (2) (118–122)	89 (4) (88–182)	131 (2) (122–140)	175 (4) (125–195)	
MF		147 (122) (81–254)					[23]

[a] First line: mean or mean ± standard deviation in μg/100 ml (number of individuals in study); second line: (range of values found)
[b] RBC = red blood cells

firmed by Evers [23] and Van Ravesteijn [24] who also found that RBC Cu decreased in spite increased whole blood Cu.

Subsequently, Brendstrup [9,25] correlated the TS Cu increase in febrile patients and return to normal in remission, with other parameters associated with disease activity. As shown in *Table 5.2*, active disease was associated with an increased TS Cu which was directly related to decreased mobility and haemoglobin (Hb) values as well as increased ESR and duration. The mean value of TS Cu in patients with normal mobility differed relatively little from the mean value found for patients with inactive joint lesions. Patients with active RA who showed clinical improvement had a pronounced fall toward normal TS Cu and ESR but these parameters remained relatively unchanged in patients showing no improvement.

Brendstrup's data also confirmed that the normal sex-related serologic differences in TS Cu were no longer apparent in active disease states because of marked increases in the concentration of this metal in both men and women as shown in *Table 5.3*. In addition, these serologic changes were most pronounced

Table 5.2. MEAN MOBILITY INDICES (MI), ERYTHROCYTE SEDIMENTATION RATE (ESR), DURATION, HAEMOGLOBIN(Hb), TOTAL COPPER(Cu) AND ACTIVITY OF DISEASE FOR PATIENTS WITH CHRONIC RHEUMATOID ARTHRITIS [25]

MI [a]	*ESR* [b]	*Duration* [c]	*Hb %*	*Cu* [d]	*Activity* [e]
1.0 (25)	10 (25) (1–36)	5 (23) (1–16)	92 (25) (77–110)	135 (24) (86–243)	19i 6a
0.97 (31) (1–0.95)	24 (31) (1–90)	6 (29) (0.5–26)	92 (31) (73–111)	152 (25) (82–319)	16i 15a
0.93 (24) (0.95–0.90)	23 (24) (4–51)	6 (21) (1–31)	91 (24) (81–114)	173 (21) (117–231)	8i 16a
0.87 (30) (0.90–0.83	39 (30) (1–116)	8 (29) (1–26)	87 (30) (62–115)	175 (26) (91–282)	4i 26a
0.80 (23) (0.83–0.75)	43 (23) (3–91)	8 (20) (1–17)	82 (23) (57–100)	203 (19) (117–261)	3i 20a
0.61 (18) (0.75–0.07)	50 (18) (10–127)	18 (18) (3–43)	81 (18) (65–104)	193 (18) (121–292)	0i 18a

[a] First line: mobility index (number of individuals); second line: (range of values included in group)

[b] First line: mm h^{-1} (number of individuals); second line: (range of values in group)

[c] First line: years (number of individuals); second line: (range of values in group)

[d] see footnote a, *Table 5.1*.

[e] number of individuals with inactive (i) and active (a) disease

Table 5.3. DISEASE ACTIVITY, SEX AND MEAN ERYTHROCYTE SEDIMENTATION RATE (ESR), DURATION OF ILLNESS, HEMOGLOBIN(Hb), TOTAL COPPER(Cu) AND MOBILITY INDICES (MI) FOR PATIENTS WITH CHRONIC RHEUMATOID ARTHRITIS [25]

Activity [e]	*Sex*	*ESR* [b]	*Duration* [c]	*Hb %*	*Cu* [d]	*MI* [a]
i	F	6 (29)	6 (28)	90 (29)	133 (28)	0.96 (29)
i	M	3 (21)	8 (18)	102 (21)	132 (17)	0.95 (21)
a	F	45 (69)	8 (63)	83 (69)	187 (62)	0.86 (69)
a	M	43 (101)	9 (94)	85 (101)	188 (88)	0.84 (101)

[a,b,c,d,e] See footnotes to *Table 5.2.*

in patients with the most active rheumatic disease. This led Brendstrup to suggest that the pathologic activity of RA could be evaluated by determining the magnitude of the TS Cu concentration. Alternatively, the rate of remission was related to the rate at which the TS Cu concentration returned to normal.

In summary, the results published from 1938 to 1953 clearly demonstrated that compared with normal healthy individuals, patients with RA had higher mean TS Cu concentrations which were 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 by diminished strength and decreased Hb values. Small sex-related differences in normal individuals were obscured by marked increases found for both male and female patients. The TS Cu which increases in association with the onset and persistance of active disease, returned to normal with remission.

It is now known from animal studies that the rise in TS Cu, is accompanied by a fall in liver Cu concentrations. Since serum Cu containing components are synthesized in the liver and appear in serum following the onset of disease, it seems reasonable to suggest that this is a physiological response to this disease.

Low or normal serum Cu concentrations shown in the range of values obtained for some patients with RA deserves some comment. These values may be the result of a failure of this aspect of the physiologic response due to depletion of liver Cu stores. Depletion may result from increased turnover resulting in Cu excretion [26,27] and a failure to replete these stores either through loss of appetite or inadequate diet. Failure of serum Cu to increase maximally as a physiological response to disease could lead to chronic disease.

Following the reports by Brendstrup in 1953, there was little published concerning changes in blood essential metal concentrations associated with RA until 1965 when interest in this topic was revived. This renewed interest has con-

tinued to the present and results obtained during this period are presented in *Table 5.4.*

In 1965 Plantin and Strandberg [28] reported results which confirmed the report by Van Ravesteijn [24] that total whole blood Cu was elevated in RA. Analyses of whole blood for Cu were shown to give values which were essentially the same, regardless of disease activity. Thus analysis of whole blood for Cu did not provide useful diagnostic or prognostic information concerning the activity of this disease.

The earlier observations concerning Cu in RA were extended by Niedermeier [29] with the report that the increase in TS Cu was due to an increase in ceruloplasmin (Cp) and non-Cp Cu concentrations. Since Cp contains 0.34% Cu, the observed mean increase in Cp represented an increase of 34 μg 100 ml^{-1} while the mean non-Cp Cu increased only 11 μg 100 ml^{-1} of serum. The increased synthesis of Cp in response to RA was subsequently confirmed by Koskelo, Kekki, Virkkunen, Lassus and Somer [30] and shown to be related to disease activity [27]. Patients with severe to moderately active RA had significantly ($P < 0.05$) accelerated daily ceruloplasmin turnover-rates. 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 state, a correponding increase in the elimination-rate [27]. Turnover studies with γ_{SS}-globulin also revealed an accelerated synthesis of this protein in the majority of patients with RA.

Lorber, Cutler and Chang [31] were the first to report a statistically significant elevation of TS Cu concentrations in RA when compared with age-matched normal individuals. However, they found that almost the entire TS Cu content of normal individuals was bound Cp. This was corroborated in a second group of normal individuals with the demonstration that TS Cu contained only a small amount of non-Cp Cu (7 μg 100 ml^{-1}), which was much smaller than that reported by Niedermeier [29] (24 ± 16 μg 100 ml^{-1}). In addition, they found that their population of RA patients had a large mean non-Cp Cu concentration (103 μg 100 ml^{-1}), which was much larger than that reported by Niedermeier [29] (35 ± 25 μg 100 ml^{-1}). Based upon these results, it seems reasonable to interpret that the large increase in non-Cp Cu is an integral part of the physiological response to active disease in these patients.

The results of Lorber, Cutler and Chang [31] were questioned by Sternlieb, Sandson, Morell, Korotkin and Scheinberg [32], based upon their analyses of sera from RA patients which gave a mean non-Cp Cu concentration of only 9 μg 100 ml^{-1}. Lorber [33] suggested that this observation was due to the low TS Cu concentration obtained by Sternlieb, Sandson, Morell, Korotkin and

Table 5.4. ALTERED COPPER (Cu) CONCENTRATIONS IN RHEUMATOID ARTHRITIS

Sex	*Copper* [a]		*Ceruloplasmin* [c]		*Non-CpCu* [a]		*References*
	normal	*arthritic*	*normal*	*arthritic*	*normal*	*arthritic*	
MF	99 [b] (70)	174 [b] (46)					[28]
MF	148 ± 25 (19)	192 ± 34 (21)	36 ± 9 (19)	46 ± 11 (21)	24 ± 16 (19)	35 ± 25 (21)	[29]
MF			37 ± 5 (58) (24–50)	65 ± 18 (31) [i] (41–131)			[30]
MF			40 ± 6 (13) (27–48)	59 ± 14 (7) [i] (49–88)			[27]
?	96 (89) (60–132)	168 (10) (109–276)					[44]
MF	139 (13) (117–161)	181 [g] (20) (161–201)					[31]
MF	119 (4) (107–137)	248 (5) (210–338)	33 (4) (28–42)	41 (5) (38–52)	7 (4) (0–12)	103 (5) (75–156)	[33]
?		150 (7) (132–205)		46 (7) (42–61)		9 (7) (0–20)	[32]
MF	97 ± 32 (105)	110 ± 29 [b] (105)					[39]
M	99 (9) (73–171)	96 (14) (77–122)	27 (9) (18–52)	26 (14) (20–43)			[41]
F	107 (13) (73–146)	107 (14) (73–146)	31 (13) (20–47)	31 (14) (20–44)			[41]

MF	104 ± 1 (1)	266 ± 85 [g] (27) (66–392)					[40]
M		254 ± 10 (7)					[40]
F		271 ± 79 (20)					[40]

Sex	*SFCu* [d]		*SFCp* [e]		*SF Non-CpCu* [d]		*References*
MF	50 ± 14 (6)	107 ± 22 [j] (23)	4 ± 2 (6)	24 ± 7 [j] (23)	35 ± 14 (6)	26 ± 15 (23)	[29,39,43]
MF	28 ± 16 [f] (50)	85 ± 21 [i] (50)					[44]
?	34 [f] (7–73)						[44]
		98 (12) (66–149)					
?	43 (6) (22–59)						[44]

[a] First line: mean or mean ± standard deviation in μg 100 ml^{-1} of serum (number of individuals in study); second line: (range of values found)

[b] mean in μg 100 g^{-1} or 100 ml of whole blood (number of individuals in study)

[c] First line: mean or mean ± standard deviation in mg 100 ml^{-1} of serum ceruloplasmin(Cp) (number of individuals in study); second line: (range of values found)

[d] First line: mean or mean ± standard deviation in μg 100 g^{-1} of synovial fluid (SF) (number of individuals in study; second line: (range of values found)

[e] mean or mean ± standard deviation in μg 100 g^{-1} of synovial fluid(SF) (number of individuals in study)

[f] sample taken post mortem

[g] $P < 0.05$

[h] $P = 0.002$

[i] $P < 0.001$

[j] $P < 0.01$

Scheinberg [32] and attributed that to the methodology used to obtain their result.

However, the different analytical procedures used by these workers [29,31,32], while perhaps accounting for small differences, do not fully account for the large differences in the 3 sets of data. Alternatively it is suggested here that it may be possible to accomodate the observed differences by taking into account disease activity in the three populations studied. According to reports published up to 1953, the second group of RA patients studied by Lorber, Cutler and Chang [31] had the most active disease since these patients had the highest TS Cu concentration (248 μg 100 ml^{-1}). Since the patients studied by Niedermeier [29] and Sternlieb, Sandson, Morell, Korotkin and Scheinberg [32] had TS Cu concentrations of 192 and 105 μg 100 ml^{-1} respectively, these patients had less active disease. All 3 groups of patients had about the same Cp concentration which is 15 to 20% larger than normal values. Based upon the increases in non-Cp Cu, it appears that the patients in the second group studied by Lorber, Cutler and Chang [31] had more active disease than those studied by Sternlieb, Sandson, Morell, Korotkin and Scheinberg [32]. This last group had either inactive disease, were in remission, or were no longer physiologically responding to the disease processes.

Lorber, Cutler and Chang [31] concluded that the elevation in TS Cu associated with RA was primarily due to an elevation on non-Cp Cu and suggested that this Cu might serve as a deleterious sulphhydryl group oxidizing agent, denaturing haemoglobin, disrupting RBC and lysosomal membranes, resulting in the release of tissue lytic enzymes. They further speculated that the beneficial effects of certain chelating agents such as penicillamine are attributed to their Cu chelating action which presumably promoted the excretion of Cu. This was used to account for the beneficial effects of penicillamine resulting in lowered TS Cu, increased serum sulphhydryl content, and decreased rheumatoid factor titres in patients receiving penicillamine. However, the more recent observation by Chayen, Bitensky, Butcher and Pouler [34] that Cu stabilizes lysosomal membranes, perhaps via sulphhydryl group oxidation, and as a result decreases the ratio of free *versus* bound lysosomal enzymes is contradictory. Also, the mechanism of action of penicillamine and other drug chelating agents which appear to lower serum Cu by bringing about remission has been suggested to be the result of Cu-drug complex formation *in vivo* which facilitates remission by promoting tissue utilization of Cu rather than promoting excretion of Cu [35–38]. Based upon these considerations, it seems reasonable to interpret the large increase in non-Cp Cu observed by Lorber, Cutler and Chang [31] as an integral part of the physiological response to active disease which facilitates remission rather than causing adverse effects *via* sulphhydryl oxidation.

In a subsequent study of TS Cu, Niedermeier and Griggs [39] found TS Cu

concentrations which were unusually small and close to normal. This raises the question as to whether or not many of the patients in this group had less active disease, were in remission, or were no longer able to respond to the disease process and had chronic rheumatic disease. These suggestions are based upon the results of Brendstrup [25] and the recent findings that RA patients at or near the peak activity of their disease had 2 to 3 times the normal TS Cu levels [40]. These recent data also provided confirmation of the absence of a significant difference between the levels found for men and women with active RA.

Recently Bajpayee [41] questioned previous reported increases of Cu and Cp concentrations in sera of patients with RA. His data with plasma from both male and female patients who had a diagnosis of RA, demonstrated that total plasma Cu and Cp concentrations were the same as the concentrations found for his normal male and female populations. Based upon these results, it was suggested [40] that the patients in his study also had inactive disease, were in remission, or were no longer able to respond to the disease process and had chronic rheumatic disease.

Bajpayee [41] had suggested that the well-known sex difference (female to male ratio 2 : 1) in populations of arthritic patients may have accounted for elevated levels of Cu and Cp due to the oestrogen-mediated increase in plasma Cu concentration in the female subgroup of his RA population. However, his data did not show that women, who were not known to be on oestrogen therapy, had significantly different levels of Cu or Cp from men. Also, the TS Cu concentrations for patients with active disease [25,40] were found to be much greater than the reported [41] estrogen-induced increase in TS Cu.

Also shown in *Table 5.4* are the concentration changes of Cu in synovial fluid (SF) of RA patients. Following the qualitative observation by Schmid and MacNair [42] that Cp was present in SF, Niedermeier, Creitz and Holley [43], Niedermeier [29] and Niedermeier and Griggs [39] quantitated its presence and alteration in RA. A significant increase in total SF Cu was shown to be due to a large increase in SF Cp while the non-Cp Cu concentration decreased. Increases in Cp concentration appeared to parallel the duration of disease.

This increase in SF Cu was confirmed by Bonebreak, McCall, Hunder and Polley [44] (1968) who found that, in addition to an increase in TS Cu and total SF Cu, SF from RA patients contained 2 Cu-containing components as compared with only one in SF from non-inflamed joints. Ceruloplasmin was identified in all SF and the other, found only in inflamed joints, was suggested to be a Cu-albumin complex.

In summary, the results reported since 1965 have increased our understanding of essential metal metabolism associated with RA. Marked increases in TS Cu were confirmed in patients with active disease. The increased rate of synthesis and accelerated turnover-rate of Cp was found to be directly related to disease

activity. Copper was also found to increase in synovial fluid of RA patients and ceruloplasmin which accounted for most of the SF Cu increase was suggested to increase with increasing duration of disease. Accelerated rates of Cp synthesis and turnover, marked increases in TS Cu and an accumulation of Cp Cu in SF can all be interpreted as multifacited physiological response to inflammatory disease.

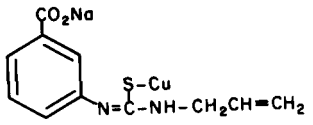
Unfortunately, some of the work done since 1965 did not take into account the results which were reported from 1938 to 1953 nor the possibility that these results had physiological significance. Consequently, the changes in Cu metabolism were interpreted as pathological. It is suggested here that if these changes in Cu-requiring tissue components are at least considered to be physiological and correlated with disease activity, duration, severity, pain, ESR, Hb levels, oedema and immobility, it may be possible to achieve a better understanding of rheumatic disease.

TREATMENT OF RHEUMATOID AND OTHER DEGENERATIVE CONNECTIVE TISSUE DISEASES WITH COPPER COMPLEXES

A historical review of the use of Cu complexes in the treatment or rheumatoid and other degenerative connective tissue diseases has been published [45]. This review provides an account of the therapeutic results obtained with Cu complexes used from 1940 to 1971 for the treatment of 1500 patients with acute or chronic rheumatoid arthritis, rheumatic fever, ankylosing spondylitis, staphlococcal spondylitis, gonococcal arthritis, chronic gouty arthritis, chronic polyarticular synovitis, coxitis, disseminated spondylitis, arthritis with psoriasis, Reiter's syndrome, lupus erythematosus, sarcoidosis, arthrosis deformans, erythema nodosum sciatica (with and without lumbar involvement), cervical spine-shoulder syndrome, lumbar spine syndrome or osteoarthritis. Even though these clinical applications lacked more modern control studies, the results revealed Cu complexes were useful anti-arthritic drugs. Treatment with these Cu complexes produced no recognized Cu-induced toxicity and there was no gastrointestinal irritation, which is a common toxicity associated with the use of modern anti-arthritic drugs.

Prior to 1940 copper oxide has been successfully used in the treatment of tuberculosis. However, in 1940, several additional Cu complexes were reported to have anti-tubercular activity and one of them, the Cu complex, sodium 3-(allylcuprothioureido)benzoate (allocupreide sodium, Cupralene, 19% Cu, 1) was suggested to be superior to gold (Au) therapy of tuberculosis [46–48].

Following this report, Fenz [49] used allocupreide to treat rheumatoid arthritis (RA). Modest results were obtained with intravenous (i.v.) injections of



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low doses of allocupreide but exceptional results were claimed with some patients given larger doses 3 times per week. Treatment was completed after 10 to 14 injections and corresponded to 0.54 to 1.24 g of Cu. 'Excellent results' were achieved in 21% of the cases, 41% had received 'important benefits', compared with 31% who had 'improved moderately' and 7% who had experienced no effect [49,50]. Unfortunately, the terms excellent, important and improved moderately were not defined as to change in objective or subjective symptoms of disease.

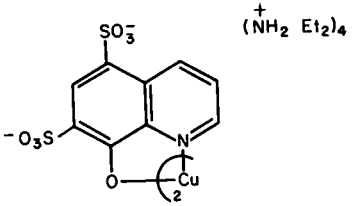
Fenz also reported that therapy with allocupreide also brought about remission of the anaemia associated with RA. This was consistent with the observations of others who found that anaemic girls working in a Cu mine were soon relieved from their anaemia and from what is now known to be a Cu-dependent iron mobilization process required for haemoglobin synthesis [1,51,52]. Fenz also reported that toxic side-effects, such as those that occur relatively often with Au therapy, were not observed with Cu therapy.

Forestier [53] also recognized that Au therapy was associated with a high risk of toxic manifestations and in his search for drugs of lesser toxicity began to use allocupreide in 1942. In 1944, he published preliminary results which were patterned after his 20 yr of clinical experience with the use of Au compounds. His first group of patients consisted of 33 RA, 4 ankylosing spondylitic and 6 osteoarthritic patients [53]. Of these, 45% or 14 were reported to have had 'very good' results, 20% or 6, had 'favourable' results and 35%, 11, had 'moderate improvement' which was consistent with the results obtained by Fenz [49,50] but these clinical classifications were also undefined. Subsequently, Forestier and Certonciny [54] found that increasing the dose so that 47.5 to 95 mg of Cu was given per i.v. injection and increasing the duration of therapy as well as total dose per series (380 to 950 mg of Cu) increased the proportion of patients receiving 'very good' and 'favourable' results.

Separation of these patients into groups by disease category illustrated that not all patients were equally benefited with allocupreide. More patients (38%) with RA had 'very good' responses when the duration of their disease was less than one year. There were no patients in the 'moderate' effect group and only 1 (8%) in the 'no effect' group when the disease was of shorter duration. When the disease was of more than 1 yr in duration only 1 patient (4%) obtained 'very

good' results while 8 (35%) had 'good' results, 9 (39%) had 'moderate' results and 5 (22%) experienced no beneficial effect. All patients with chronic polyarticular synovitis were favourably affected but none of the responses were rated as 'very good'. No beneficial effect was observed in the disease categories of ankylosing spondylitis, acute articular rheumatism, or monoarthritis. In contrast to these, a 'good' response was observed with one patient who had staphlococcal spondylitis. In summary, these data demonstrated that 72% or 56 individuals received some benefit with allocupreide therapy while 28% or 14 patients received no beneficial effects.

In a continuing search for more effective therapy, Forestier, Jacqueline and Lenoir [55] in 1948 reported their results with a new Cu complex, bis(8-hydroxyquinoline di(diethylammonium sulfonate)) Copper(II) (cuproxoline, Dicuprene, Cuprimyl, 2). Although this compound had been found by Michez



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and Ortegal [56] to be less effective orally, Forestier, Jacqueline and Lenoir [55] considered cuproxoline to be somewhat superior to allocupreide because it was less irritating and could be given by both i.v. and intramuscular (i.m.) routes of administration.

Cuproxoline (6.5% Cu) mixed with novocaine was given i.m. or i.v. 2 or 3 times a week (32.5 to 65 mg of Cu), with a total dose of 390 to 780 mg of Cu per series. As in Au therapy, this Cu compound was given in series of injections, with rest periods between series. Intervals between series were not to exceed 1 mth between the first 2 series, and 2 to 3 mth between following series. Any one series of injections was not to exceed 15 to 20 doses and treatment was to be continued for several series to assure that all signs of active arthritis disappeared and ESR returned to normal.

The results with cuproxoline therapy were only 'good' when it was used to treat RA which had been active for less than a year, but these were obtained even when the patient had become resistant to Au therapy. From the results obtained in this study, it appeared that patients who had RA for longer than a year were benefited the most with cuproxoline. Initial patient therapy with cuproxoline showed that 3 (23%), 7 (54%), 2 (15%) and 1 (8%) obtained 'very

good', 'good', 'moderate' and 'no' effect respectively. However, some patients treated with cuproxoline following toxic or intolerance reactions to Au therapy were also benefited. Two (10%), 9 (50%) and 2 (10%) respectively progressed to the first 3 stages of relief. Unlike allocupreide, cuproxoline therapy produced 'very good' results in 2 (6%) of the patients with ankylosing spondylitis and 'very good' results in a patient with disseminated spondylitis. In addition, of 4 patients with gonococcal arthritis, 2 (50%) achieved 'good' effects with one each in the 2 lesser effects categories. Treatment of 3 patients with chronic polyarticular gout produced 'very good' results in 2 (67%) and 'good' results in 1 (33%) of these patients. Treatment of 4 patients with chronic polyarticular synovitis produced 'very good' results in 2 (50%) and 'good' or 'moderate' results in the others. Two patients with monoarthritis obtained 'very good' or 'good' results. The least affected category was coxitis; of the 2 patients treated only one obtained 'moderate' relief. Christin in a personal communication to Forestier [54] reported that patients with both RA and psoriasis, received simultaneous ameloriation of both articular and skin lesions when treated with cuproxoline.

In 1949 and 1950, Forestier presented summary comparisons of results he, Certonciny and Jacqueline [57–60] had obtained with Cu and Au therapy of RA and other degenerative diseases. In RA of less than one year in duration, Cu therapy (particularly allocupreide) was more effective and less toxic than Au therapy. When Au therapy was tolerated, it was preferred for patients who had RA for more than one year in duration.

However, Cu complexes were far superior to Au therapy in treating chronic polyarticular synovitis with effusion [59]. This clinical entity, associated with a high ESR and sometimes marked anemia was found to be resistant to Au therapy. In 80% of their patients, effusions entirely or nearly cleared after 2 or 3 series of injections.

Thirty-seven patients with chronic polyarticular gout responded favorably to small doses of Cu complexes, as they did with Au therapy. Twenty-one given i.m. injections for 2 to 3 series enjoyed remarkably rapid elimination of signs of gouty polyarthritis, and 8 others were ameloriated in 4 to 5 series. In 8 others, which were more recent cases, i.v. or i.m. therapy was successful when continued [60]. 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 Cu therapy which was by far superior to Au therapy and brought about astonishing improvements in both skin and joint lesions [57]. Beneficial use of Cu therapy in infectious arthritis of known or unknown origin or in rheumatic fever was less certain. A decrease in ESR was found to correspond with clinical improvement over a period of 2 or 3 mth [57].

In summary, Cu therapy administered according to recommended doses was effective and did not as a rule cause untoward reactions. Onset of improvement was immediate, definite and progessive in most patients. Twelve patients were followed for over 2 yr and showed no relapse. It was felt that this period was long enough to make it unlikely that these were spontaneous remissions. Erythrocyte sedimentation rates returned to normal faster in patients with RA of less than 1 yr duration than in those with chronic disease. 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 signs of marrow depression were noted in these 8 patients. Copper compounds used to continue therapy in patients who were resistant or intolerant to Au therapy were found to be advantageous since they were used without ill-effects in the event of Au therapy-induced dermatitis (rashes or stomatitis) or nephritis (albuminuria).

Results of a study using Cu morrhuate therapy of RA were also reported in 1950 by Graber-Duvernay and Van Moorleghem [61]. This mixture of Cu complexes, formed with the fatty acids obtained from cod liver oil, was given i.v. as a colloidal suspension along with novocaine. Of 117 RA patients treated with this mixture, only 30% were benefited.

In 1950 Tyson, Holmes and Ragan reported their evaluation of allocupreide in what were described as 27 typical RA patients [62]. The first group of 20 patients treated in this study were reported to be severe RA patients who had their disease for 2 months to 15 yr. These patients had been either resistant to or intolerant of Au therapy. In the first course of treatment, doses were used as recommended by Forestier for patients with disease of less than 1 yr duration. Only 2 patients, who had their disease for two mth to 1 yr, obtained subjective improvement according to criteria of the American Rheumatism Association (ARA) for marked improvement or remission. All patients tolerated the total course of therapy without toxicity.

In correlating duration of disease in patients who were subjectively improved, those who had benefited most had their disease for shorter durations. Consequently 7 patients with unquestionable RA of less than 1 yr duration were treated in addition to the original 20. Individual and total doses of allocupreide given to this group were increased to non-recommended doses. The therapeutic results obtained in this study were reported to have been essentially similar to those with the smaller doses. However, it was found that none of these patients, no matter how long or short the duration of their disease, showed any significant improvement throughout the average follow-up period of 1 yr. Although Forestier had recommended multiple shorter series of injections before evaluation, these authors were forced to disontinue therapy after one prolonged series when severe toxicity was encountered with the larger doses. Based upon these

effects, Forestier had recommended small single doses and smaller total doses per series of injections of this Cu complex.

These authors also reported, for the first time, that an unusual side-effect had been noted. Small doses of allocupreide regularly caused patients to experience a taste and smell suggesting 'garlic or Musterole', 8 to 12 s after i.v. injection. In some instances, when a small quantity of allocupreide escaped from the vein into the subcutaneous tissues, a very severe and painful cellulitis resulted. In no instance did suppuration or necrosis occur and in 3 to 10 days the cellulitis subsided.

Whereas during the early course of allocupreide treatment with smaller doses administered i.v. twice a week, toxicity was rare, in the later stage using larger doses toxicity was the rule. On discontinuing Cu therapy all symptoms of toxicity disappeared and within a month blood counts returned to normal.

In the following year, Kuzell, Schaffarzick, Mankle and Gardner [63] published their study of Cu therapy in a variety of rheumatoid and degenerative diseases using the criteria of Steinbrocker, Trager and Batterman [64] and the ARA. Cuproxoline and allocupreide were used to treat patients who had RA, RA with psoriasis, Reiter's Syndrome, ankylosing spondylitis and chronic gouty arthritis.

Of the 31 RA patients treated, only 6 progressed to Grades I and II. Therefore, only 20% appreciably improved and that was interpreted to be less than satisfactory, or apparently without benefit. However, patients who had improved had less severe stages of RA. Five patients or 56% of those treated who had less severe or early stage of psoriasis with RA also responded with Grade I or II improvement without skin lesion progression or excerbation. Slightly more improvement in these patients than those arthritics without psoriasis was suggested to merit further study. Two of the 3 patients with ankylosing spondylitis showed responses of Grade I and III. However, these patients received other drugs in addition to Cu complexes and this prevented any conclusion concerning the efficacy of Cu therapy. Three patients with Reiter's syndrome were 'apparently cured' with cuproxoline. In each of these, 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. Thirteen of 18 patients with chronic gouty arthritis had a Grade I, II or III response to Cu therapy and this result was also viewed as worthy of study. However, although this does seem to be inconsistent, these authors concluded that based upon these limited successes, the Cu complexes were therapeutically ineffective.

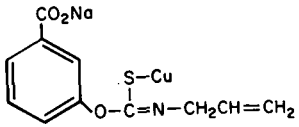
According to Kuzell, Schaffarzick, Mankle and Gardner [63] the toxicity of these substances was slight, infrequent and of a mild transitory nature, in con-

trast to the toxicities observed by Tyson, Holmes and Ragan [62]. They also noted the garlic-like odour of the breath with allocupreide which persisted in some instances for as long as 10 h. When allocupreide escaped from the vein, intense pain and necrosis followed.

Cuproxoline produced pain on i.m. injection so that in the later phases of the study this material was given i.v. There was no garlic-like odour of the breath with cuproxoline. Three patients developed amenorrhea while taking cuproxoline but menstruation was resumed after cessation of medication, and one became pregnant. This non-gravid amenorrhea was suggested to reflect some endocrine affect of cuproxoline.

Consistent with an earlier report of Forestier and Certonciny [59] that the LD_{50} values determined in rats for allocupreide and cuproxoline were 160 mg kg^{-1} and 120 mg kg^{-1} respectively (route of administration not given), Kuzell, Schaffarzich, Mankle and Gardner [63] reported rat LD_{50} values of 160 mg kg^{-1} (intraperitoneal) and 376 mg kg^{-1} (i.m.) for allocupreide, and 126 mg kg^{-1} following i.m. administration of cuproxoline.

In 1955, O'Reilly [65] reported results of a single study with i.m. sodium *m*-(*N*-allylcuprothiocarbamide)benzoate (cuproally-thiourea-sodium benzoate, Alcuprin, 3), an analogue of allocupreide and cuproxoline, in 10 patients with



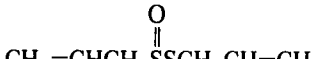
(3)

well-established RA of 1 to 13 yr duration. Four patients had had crysotherapy. All patients had 2 courses of treatment in a single series. Each course was comprised of 10 doses cuproally-thiourea-sodium benzoate or cuproxoline.

While no toxic effects were observed, only 2 patients experienced slight improvement. Two patients in this group had psoriasis but these patients received no benefit from Cu therapy. It was recognized that greater beneficial effects might have been expected in a clinical trial where cases of short duration were not excluded from the group of patients studied.

The curious initial report by Tyson, Holmes and Ragan [62] that allocupreide produced a garlic-like odour on the breath which was confirmed by Kuzell, Schaffarzick, Mankel and Gardner [63] but never mentioned by Fenz [49,50], Forestier, Certonciny, Jacqueline, and Lenoir [53–55,57–60] or O'Reilly [65] prompted a search of the literature for compounds which were responsible for

the odour of garlic. Three such compounds were found; allicin (4), garlic oil (allyl sulphide) (5), and alliin (6).



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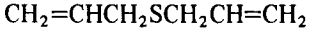

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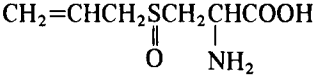


(6)

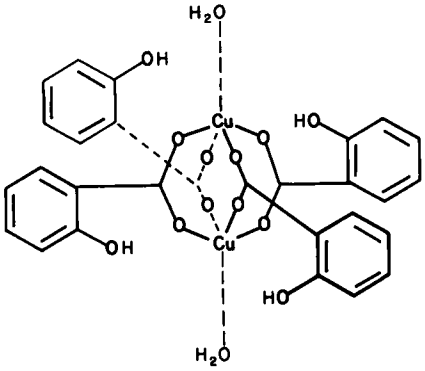
All 3 of these compounds and allocupreide contain an allylmercapto (CH_2=$CHCH_2$S-) group. This observation suggests that the garlic taste and odour were a result of the use of impure or decomposed allocupreide. Presence of (4) in an ampoule of allocupreide may have been the result of chemical decomposition via intramolecular rearrangement of the starting thiourea, followed by oxidation of the resultant allylmercaptan by Cu^{2+} to the allyldisulfide and subsequent air oxidation to the monosulfoxide (4). If the material injected into those patients was not allocupreide, having the structure as shown (1), then this might also partially explain the unsatisfactory therapeutic and toxicologic results.

Studies of the mechanism of action for allocupreide were reported by Wintrobe, Cartwright, and Gubler [66] in 1953. They found that 250 mg of allocupreide (50 mg of Cu) given i.v. to normal adults caused a rapid increase in plasma and RBC Cu, which did not occur with oral administration, followed by a decrease to normal levels in 4 h. It was suggested that the disappearance from plasma was associated with uptake by the reticulo-endothelial system prior to erythropoesis. This was consistent with the earlier observations of Heilmeyer, Keiderling and Stuwe [6,7] that serum Cu increased in patients with RA but returned to normal with remission of their arthritic disease and associated anaemia, as well as the anti-anaemic effects of allocupreide reported by Fenz [49]. These observations and suggestions were consistent with the known requirements of Cu for iron utilization in haemoglobin synthesis [1,51,52].

Although allocupreide and cuproxoline were shown to be effective and in some instances superior to Au therapy of rheumatoid and other degenerative connective tissue diseases, nothing more was published concerning these Cu complex drugs after 1955.

However, in 1950 Hangarter had begun his research with his newly developed salicylic acid Cu complex (Permalon, SACC). The structure(s) of the Cu complex(es) of salicylic acid in SACC are not known with certainty but 2

possible structures are (7) and (8). The following is an account of this work at



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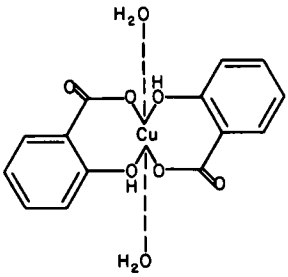


(8)

Bad Oldesloe Hospital which continued until 1971, when he retired and the manufacture of SACC was discontinued.

Historically, the therapeutic potential of Cu for the treatment of rheumatic diseases was independently recognized by Hangarter in 1939 when he learned that Finnish Cu miners were unaffected by rheumatism as long as they stayed with the mining industry [67]. This was particularly striking since rheumatism was widespread in Finland and workers in other industries and other towns had more rheumatic disease than these Cu miners.

Hangarter attributed this effect to Cu; this suggestion was consistent with the results that serum Cu increased in patients with rheumatic disease and infections and returned to normal with remission [6,7]. Therapeutic results with inorganic Cu alone were found by Hangarter to be comparable with those of Au therapy, although Cu treatment was associated with fewer side-effects [68]. Based upon these results and his experience with i.v. salicylic acid therapy of RA, he developed SACC with the assistance of Dr. Reiser of the Albert Chemical Company. SACC was an aqueous mixture of $CuCl_2$ and sodium salicylate. One 20 ml ampoule contained 2.0 g (12.5 mmole) of sodium salicylate and 2.5 mg (39 μmole) of Cu.

From 1950 to 1954, SACC was administered by i.v. injection. Daily injections of 1 ampoule over an average period of only 8 to 14 days produced beneficial results. In cases of therapeutic success as defined by the American Rheumatism Association and Steinbrocker, Trager and Batterman [64] (remission of fever, alleviation of pain, increased mobility, inhibition of exudative joint effusions and a decrease in the ESR in various stages [64] of rheumatic diseases),

the initial period of medication was followed by one or several days without treatment [67]. A sudden discontinuation was not advised, since prolonged administration guaranteed a better and longer-lasting effect.

Unlike results obtained with allocupreide and cuproxoline in the treatment of patients with acute rheumatic fever (ARF), SACC produced dramatic improvement. A single injection (20 ml), markedly decreased pain, reddening and swelling of effected joints. These ARF patients were completely free of pain and fever, and had increased mobility after 1 or 2 additional administrations. Because erythrocyte sedimentation rates decreases more slowly, treatment was continued until normal ESR values were achieved. No relapses were recorded during the observation period. On average, 20 injections (50 mg of Cu) were necessary to achieve a symptom free * classification for 100% of his patients.

Effects achieved by i.v. therapy with Cu salicylate in cases of rheumatic carditis were also remarkable. After transient, highly inflammatory acute articular episodes accompanied by high fever, a typical endocarditis or myocarditis had developed in his original group of 11 patients. In these severe cases, all signs of articular inflammation completely subsided after 3 or 4 injections (7.5 to 10.0 mg of Cu) 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 favorable objective clinical course of the disease, ESR values improved but treatment was continued until normal ESR values were achieved.

The broadest clinical experience with SACC therapy was gained in the treatment of RA. Of the original group of 27 patients; 13 (48%) progressed to a symptom free classification, 13 (48%) progressed to the improved ** classification, and only 1 patient was unaffected † by SACC therapy. Many of these patients had been previously ineffectually treated with a variety of antirheumatic drugs, including salicylates, Au compounds and corticoids but they responded to SACC in a step-by-step fashion.

The initial effect following the first injection was rapid alleviation of pain as well as improved articular mobility, even in patients with severe deformities, due

* 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.

** 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.

† 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.

to decreased articular swelling resulting from extensive joint effusions. Erythrocyte sedimentation rates were also characterized as dropping slowly but steadily even though the objective clinical picture indicated a rapid complete remission. 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 confirmed by Fahndrich [69] in 1952 and Broglie [70] in 1954.

Four patients with sarcoidosis responded in a similar manner to SACC therapy but they only achieved the status of 'improved'.

SACC therapy of 5 patients with arthrosis deformans was least successful. Pain and mobility were influenced to such a slight degree that this therapy could not be considered superior to conventional forms of treatment, although 3 of the 5 patients progressed to the 'improved' category.

Two patients suffering from a classical erythema nodosum accompanied by very painful arthralgia were treated with SACC and both achieved complete remissions. This was particularly significant since experience had shown that this type of rheumatic disease was very resistant to treatment. After 2 or 3 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 therapy, ESR were 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), SACC was much more effective and rapidly acting.

Rheumatic neuritis (sciatica and facial) also responded to SACC therapy with rapid alleviation of pain and all 5 of the treated patients reached the symptom free classification. However, as far as motor impairment and its duration were concerned, the drug'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 Cu in his preparation of Cu salicylate. It was known that to achieve the same therapeutic success with only i.v. sodium salicylate therapy, a serum level of at least 25 mg% was necessary. To achieve this serum concentration with SACC a patient had to receive a total dose of 12 g of salicylic acid, 6 SACC injections, divided into equal administrations throughout a 24-h period. Since this much SACC had not been given and the salicylate level in the blood reached a peak value of only 20 to 24 mg% and dropped to 5 to 8 mg% within 24 h, he concluded that SACC's effect was not due to salicylic acid alone. It is reasonable to suggest that the marked therapeutic efficacy of SACC was due to the presence of a Cu salicylate complex or complexes.

Hangarter found no systemic toxic or noxious side-effects in these initial studies with SACC even on long term i.v. administration. Aanalyses of blood components, 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 2 injections per day were given. It was particularly significant that gastrointestinal disturbances and cerebral toxic reactions, which were investigated, were not found. A few patients experienced transient sciatic pain during i.v. injection of the first SACC preparations but this effect was no longer observed after subsequent pharmaceutical development of the preparation. However, occasionally i.v. administration was accompanied by pain or transient injection site reddening when therapy was long-term and the veins were in poor condition or the 20 ml in the ampoule was injected too rapidly.

Because of this, SACC was administered to all patients by i.v. infusion from 1954 to 1971. This mode of administration achieved equally good results with fewer treatments and without causing venous irritation. All patients received 500 ml of normal saline containing 3 to 4 ampoules of SACC per infusion (7.5 to 10.0 mg of Cu). In order to avoid intimal irritation with slow infusions, 2 ml containing 0.4 mg of novocaine were routinely added to the infusion solution. This dose of novocaine was much less than the usual therapeutic dose of 4.0 mg kg^{-1} of body weight and was not considered to be a systemic analgesic dose.

Hangarter pointed out that the number of infusions necessary to achieve a therapeutic success 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, SACC was effective in diseases of all durations when given in the prescribed manner. In general, 6 to 8 infusions (45.0 to 80 mg of Cu) at intervals of 2 to 4 days sufficed. These infusions were well-tolerated despite the high dosage level. A transient nausea accompanied by tinnitus occurred occasionally, 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 functional disturbances, usually observed at much lower oral salicylic acid doses, was again striking. With infusion therapy the blood chemistry, kidney and liver functions, blood sugar, serum electrophoresis, electrolyte metabolism, ECG and rheumatic serology were evaluated. No pathological changes of abnormal reactions were found. Nor were any cerebral, respiratory or circulatory toxicities observed.

Clinical results of i.v. infusion therapy corresponded to the successful clinical results previously described for those patients treated with daily i.v. injections of 20 ml of SACC [68]. With infusion therapy, 78 ARF patients or 100% become symptom free. Acute symptoms subsided, in many cases almost immediately, after 1 or occasionally after 2 to 3 infusions (20 to 30 mg of Cu). Average duration of treatment was only 14 to 18 days. This 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 [64] of RA received SACC by i.v. infusion [71]. Of these, 403 (65%) achieved symptom free status, 143 (23%) achieved improved status and 74 (12%) remained in the unaffected category when the supply of SACC was discontinued. On an average, the number of infusions ranged between 6 (45 to 60 mg of Cu) and a maximum of 10 (75 to 100 mg of Cu). With RA as well as agressive forms of polyarthritis, patients progressed to a symptom free classification in a step-by-step manner. The initial effect, evident after 1 or 2 infusions, was a marked alleviation of pain and improved mobility which 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 associated articular exudations. Erythrocyte sedimentation rate which was the most important indicator of all subjective pathological processes was characterized by a slow and steady drop, parallel to the decrease in rheumatic serology, even though the objective clinical picture indicated very rapid complete remission. In many cases, a normal ESR was reached only after hospital discharge in out-patient follow-up studies. Latex tests remained positive longer than elevated ESR values.

No serious toxic disturbances were recorded in association with this high dose of SACC. However, there was a toxic dose limit where nausea and tinnitus appeared but in view of the rapid alleviation of pain, these toxicities were usually considered as minor side-effects by the 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 SACC [72]. These patients were generally treated with 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 to 8 infusions (40 to 80 mg of Cu) *per day* at intervals of 2 to 3 days. Of the 162 patients with these syndromes who had undergone therapy, 95 (57%) became symptom free, 52 (32%) were classified as improved with complete relief from pain, and only 18 (11%) experienced no beneficial effect.

Two hundred and eighty patients who had the diagnosis of sciatic neuritis or neuralgia with or without intervertebral disc changes underwent in-patient treatment with SACC [72]. Most of these 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. 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 and etiology appeared to be due to a slipped disc. Most patients included in this study had already undergone several years of unsuccessful orthopaedic and conventional antirheumatic therapy. Therapy designed to treat neuritis and neuralgia was also unsuccessful.

Depending on duration and severity of disease, sciatic patients received between 6 and 8 SACC infusions (60 to 80 mg of Cu) every 3 to 4 days. A total of 120 sciatica patients without lumbar involvement experienced the following results: 76 (63%) became symptom free *, 38 (32%) reached the improved classification, and the remainder, 6 (5%) obtained slightly improved results. Of the 160 sciatic patients with lumbar involvement 95 (59%) progressed to the symptom free category, 39 (24%) obtained improved status, 10 (6%) reached the slightly improved status and 16 (11%) remained unchanged.

In spite of the above mentioned diverse etiology of these diseases, SACC 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 inability to change one's position subsided within a short period. Spinal scoliosis was corrected. Neurological symptoms provoked by the disease; absence of or differences in reflexes, a positive Lasegue's sign, disturbances in sensitivity and trophism were normalized very rapidly. It was particularly interesting that 12 of the 16 patients that 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.

More recently, through Schulte's efforts, a new anti-inflammatory Cu-dependent metallo-enzyme has been developed for drug use. This durg, orgotein, has recently been shown to be safe and effective for the treatment of established osteo-arthritis when given intra-articularly into knee and hip joints in single or multiple doses [73]. Two to 20 mg given in one to 10 injections with intervals from 3 to 64 days between injections brought about long-lasting decreases in

* 'Symptom free': Disappearance of (subjective) symptoms, Lasegue's sing negative, normal reflexes with equal quality on both sides, no disturbances in sensitivity, no tenderness on pressure and mobility restored. 'Improved': Not completely relived 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.

disease activity. Of 19 patients treated, 16 experienced a decrease in pain, reduced joint effusion and increased mobility lasting for more than 90 days after termination of orgotein treatment. Stimulation of polymorphonuclear leukocyte chemotaxis, stabilization of lysosomal membranes, reduction of lysosomal membrane permeability and superoxide dismutase were implicated as modes of action for orgotein.

Following a recent disclosure that Cu complexes had anti-inflammatory activity, Walker, Griffin and Keats [74,75] evaluated the clinical efficacy of the Cu bracelet in a single blind cross-over study. Their preliminary results demonstrated that, to a significant number of arthritic patients, the wearing of the Cu bracelet appeared to have some therapeutic value. Significantly ($P < 0.01$) more of the patients who differentiated between the Cu bracelet and placebo (anodised aluminum bracelet) perceived the Cu bracelet as being more effective. Also, previous wearers of the Cu bracelet were significantly ($P < 0.02$) worse when not wearing the Cu bracelet.

Walker, Griffin and Keats [74,75] also provided evidence to show that, while the Cu bracelets were worn, the average weight loss was 12 mg per mth. Analyses of human sweat before and after the Cu bracelet was worn revealed that the Cu concentrations in sweat after the Cu bracelet was worn were higher than before it was worn. Equilibrations of metallic Cu turnings with human sweat also demonstrated that the components in sweat solubilize this metal and possibly facilitate its absorption. Subsequently Walker, Reeves, Bronson and Coleman [76] demonstrated that a Cu complex which was likely to be formed while wearing a Cu bracelet could be absorbed by perfusing cat skin with ^{64}Cu(II) $(glycine)_2$. The ^{64}Cu was found in all layers of the skin and following 6–7 h a steady state subcutaneous concentration was reached. During a 24-h period, 1 mg of the complex had perfused in the intact skin.

In the light of this and other scientific evidence which supports the observed clinical effectiveness of the Cu bracelet, Whitehouse and Walker [77] have suggested that it would be appropriate for the medical profession to reconsider the validity of the Cu bracelet in the treatment of arthritics.

PHARMACOLOGY OF COPPER COMPLEXES

Prior to 1950, the anti-inflammatory activities of Cu complexes had been exclusively studied in humans for the treatment of tuberculosis and rheumatic diseases. In 1950 Kuzell and Gardner [78] reported that the i.m. administration of allocupreide (1) favourably influenced polyarthritis produced in rats with a pleuropneumonia-like organism. However, in 1951, using this same model of experimental arthritis, Kuzell, Schaffarzick, Mankle and Gardner [63] reported

that both allocupreide and cuproxoline (2) were ineffective in preventing or 'curing' the rather variable inflammation in this model. They felt that these results were consistent with their clinical results but it seems that the allocupreide used had either decomposed or was impure (see p. 222).

A more favourable interpretation was offered by Vykydal, Klabusay and Truavsky [79]. In summarizing their results with formalin-induced rat paw oedema, they suggested that the mechanism of action of parenterally administered allocupreide was similar to that of adrenalcorticotrophic hormone. This favourable interpretation was suggested to be consistent with the clinical results of Fenz [49,50] as well as those of Forestier, Certonciny, Jacqueline and Lenoir [53–55,57–60].

Subsequently, Schubert published his accounts of the hypothermic effect of i.v. administered sodium salicylate, copper (II) chloride ($CuCl_2$) and Cu salicylate (7,8) [35,36]. Copper(II) salicylate was more effective in lowering yeast-induced rat hyperthermia than either $CuCl_2$ or sodium salicylate. The *in vivo* formation of a Cu chelate of an analogue of salicylic acid was invoked by Schubert to account for the lowering of rat body temperature in either the normothermic or hyperthermic state [75,76]. These observations provide mechanistic insight into Hangarter's [67,68,71,72] observed rapid reduction of fever in febrile patients with acute rheumatic and degenerative connective tissue diseases when they were treated with his Cu salicylate preparation (SACC). These results also account for the mild to severe chills observed by Tyson, Holmes and Ragan [62] when larger doses of allocupreide were used in their clinical studies.

In 1958, Sutter, Adjarian and Haskell [80] reported that a large oral dose of copper(I) iodide (250 mg kg^{-1}) caused a 60% reduction of the granuloma and almost completely eliminated exudation in the rat granuloma-pouch (GP) model of inflammation. However, Adams and Cobb [81] found that even larger oral doses of up to 320 mg kg^{-1} did not delay the development of ultra-violet (UV) light-induced erythema in guinea-pigs and the activity of this compound was no longer pursued.

In 1969 a patent of Laroche [82] disclosed that Cu complexes of acetic, lauric, oleic, caprylic, butyric, sebasic, lipoic, and cinnamic acids were orally effective in rats as anti-inflammatory agents at doses corresponding to 18 and 60 mg kg^{-1} of Cu respectively in the carrageenan paw oedema (CPE) and croton oil granuloma models of inflammation. It was claimed that mixtures of these complexes with clinically used antirheumatic agents would be useful in treating a variety of rheumatic diseases. However, this approach was never used in clinical therapy.

Also, in 1969, Bonta [83] reported that the mineral, basic cupric carbonate, $Cu(OH)_2CuCO_3$ (malachite) failed to cause a 50% inhibition of kaolin-induced rat paw oedema (KPE) at 20 mg kg^{-1} when given orally but did so at 20 mg kg^{-1}

when given subcutaneously (s.c.). In addition to this substance, Bonta reported that a series of Cu chelates, which were not specified, were found to be only weakly active when given orally but had pronounced anti-inflammatory activity when given s.c. in the KPE, cotton was granuloma (CWG) and GP models of inflammation.

Because the anti-inflammatory effects were throught to be regularly associated with marked tissue irritation at the injection site, Bonta suggested that these Cu compounds may not have specifically induced the anti-inflammatory effect, but that the anti-inflammatory activity was due to nonspecific tissue irritation at the sites of injection which were remote from the site of induced inflammation. In a follow-up study of malachite, he [83] found a contrasting profile of activity in the guinea-pig UV-erythema model of inflammation. Unlike the results obtained by Adams and Cobb [81], malachite caused a 50% inhibition of UV-induced erythema at 25 mg kg^{-1} when given orally but was inactive when given at the same dose s.c. In the guinea-pig, as in rats, this compound produced marked irritation at the s.c. injection site. If, in the rat oedema test, the effect of this compound was indirect and due to the s.c. irritation, this latter factor did not seem to play a role in inhibiting guinea-pig UV-erythema. Though the gastric mucosa of guinea-pigs treated orally with malachite showed harsh irritation, there was no evidence at that time as to whether or not that provided a basis for an irritant-induced anti-erythema effect. Bonta [83] suggested that in the guinea-pig, gastric but not s.c. irritation evoked a remote anti-inflammatory effect. Alternatively, he pointed out that he had no argument to rule out the possibility that in guinea-pigs, the Cu-containing substance after being absorbed from the gastrointestinal tract may have acted by virtue of its anti-inflammatory effect [83]. This possibility seems to be just as likely in rats.

However, Bonta's impression was those compounds which were not irritating were devoid of the anti-inflammatory effect. In addition, it appeared that adrenalectomy abolished their anti-inflammatory activity. Although he suggested that tissue irritation might have stressed the rats to such an extent as to activate the hypophysyl-adrenal axis to discharge endogenous corticosteroids which in turn may have exerted the anti-inflammatory effect, he felt that this was not very likely, since corticosterone – the main glucocorticoid produced by the rat adrenal – had a particularly weak anti-inflammatory effect. However, he pointed out that the possibility of an indirect corticoid-induced anti-inflammatory effect had not been ruled out [83]. Nevertheless, Bonta chose to follow the philosophical view that local tissue irritation and remote anti-inflammatory effect may have been casually connected with each other.

Support for this idea was obtained when rats were treated intraperitoneally with phenylquinone (an irritant which causes writhing) 30 min prior to subplantar injection of kaolin. There was marked inhibition of swelling usually

caused by kaolin. This indicated that the remote inhibitory effect of tissue irritation was similar to the action of corticosteroids and non-steroidal anti-inflammatory drugs. Tissue irritation with phenylquinone may have released some factor(s) which, being discharged into the blood stream, caused a suppression of the inflammatory response to kaolin. Bonta succeeded in transferring the postulated tissue factor in lyophilized peritoneal exudate to other rats. In subsequent experiments, serum of phenylquinone-treated rats was also shown to exert an anti-inflammatory effect in the KPE test; even serum from normal animals did so, though to a lesser extent [83].

In an effort to correlate all of these experimental observations, it is suggested that one of the tissue factors responsible for irritant-induced anti-inflammatory activity may be the increase in serum ceruloplasmin as well as in albumin and amino acid Cu complexes. These are known to increase in serum of man and animals in response to irritants [6,7,66], inflammation [2,3,6–8,84], and infection [2,3,66,85] as a primary response to the etiological agents. The increase of these acute phase reactants in serum is consistent with the observations that inorganic Cu and Cu complexes have anti-inflammatory activity when tissue distribution is assured by parenteral administration and with the idea that this activity is physiological in nature. This aspect of the anti-inflammatory activity of Cu complexes is considered in greater detail (see page 213).

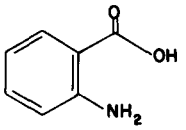
In 1966, it was found that Cu acetate [$Cu(II)_2(acetate)_4$] had potent anti-inflammatory activity in the CPE model of inflammation [37,38]. This observation along with the recognition that all of the anti-arthritic drugs can form Cu complexes suggested that Cu complexes might be the active forms of the anti-arthritic drugs [37,38,86]. The rationale for this suggestion was based upon two possibilities. First, giving $Cu(II)_2(acetate)_4$ may result in the formation of Cu complexes *in vivo* with complexing agents such as the amino acids. Second, it is known that serum Cu concentrations increase in arthritic disease [2,6] so that giving a complexing agent such as an anti-arthritic drug can result in the *in vivo* formation of a Cu complex of that drug.

At the outset, the hypothesis was tested with Cu complexes prepared with complexing agents that had no known anti-inflammatory activity of their own. This was done to avoid observing activity resulting from an active anti-inflammatory compound; in the event, there was dissociation of the complex to give what may be a known anti-inflammatory drug. If activity observed for $Cu(II)_2(acetate)_4$ was due to the amount of Cu being given, then giving Cu complexes with less Cu in them would not be as effective as given $Cu(II)_2(acetate)_4$. Initially then, Cu complexes prepared with inactive complexing agents and containing decreased quantities of Cu were used to test the hypothesis that Cu complexes had anti-inflammatory activity.

In order to test these complexes and reduce or minimize the risk of their loss

by dissociation in the stomach, they were given s.c. To further reduce or minimize the risk of losing the Cu in these complexes, they were routinely given as Tween 80 suspensions. Suspending agents such as carboxymethylcellulose, tragacanth and acacia were avoided since these are polycarboxylic acids which may have removed the Cu from these complexes. Administration of these compounds under these conditions of reduced risk of loss by dissociation throughout the course of this study gave satisfactory biological results for many Cu complexes [37]. Results obtained for only 2 of the complexes synthesized from biologically inactive complexing agents are presented here.

The anti-inflammatory activities of $Cu(II)_2(acetate)_4$, anthranilic acid (9), 3,5-diisopropylsalicylic acid (10), $Cu(II)(anthranilate)_2$ (11) and Cu(II)(3,5-diiso-



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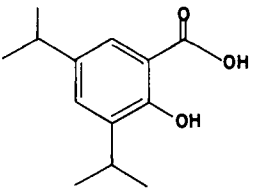

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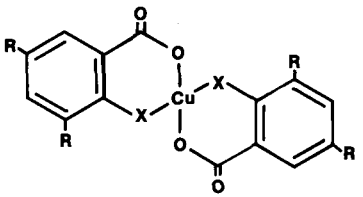


X = NH_2 and R = H or X = OH and R = -CH Me $_2$

(11)

propylsalicylate)$_2$ ($Cu(II)(3,5\text{-dips})_2$, 11), are presented in *Table 5.5*. As mentioned above, cupric acetate had been found to be active (A) in the CPE model of inflammation at 8 mg kg^{-1} but with subsequent followup testing it was inactive (I) at the initial screening doses of 100 and 30 mg kg^{-1} respectively in the CWG and adjuvant arthritis (AA) models of inflammation. Anthranilic acid and 3,5-dips were inactive (as expected) at the large initial screening doses of 200 and 30 mg kg^{-1} in the CPE and PA tests respectively. However, Cu(II)-(anthranilate)$_2$ and $Cu(II)(3,5\text{-dips})_2$ were very potent in all three models of inflammation.

The difference in effectiveness of these compounds does not appear to be

Table 5.5. COMPARISON OF THE ANTI-INFLAMMATORY ACTIVITIES OBTAINED FOR Cu COMPLEXES OF ACETIC, ANTHRANILIC AND 3,5-DI-ISOPROPYLSALICYLIC ACIDS [37]

Compound	*Carrageenan paw oedema* [a]	*Cotton wad granuloma* [b]	*Adjuvant arthritis* [c]	*%Cu*	*LD_{50} value*	*TI*	
						CPE	*PA*
Copper(II) acetate	A at 8	I at 100	I at 30	31.8	350	70	
Anthranilic acid	I at 200	NT	I at 30	13.5			
$Cu(II)(anthranilate)_2$	A at 8	A at 25	A at 1.2	18.9	750 ± 106	94	625
3,5-dips acid	I at 200	NT	I at 30				
$Cu(II)(3,5\text{-dips})_2$	A at 8	A at 5	A at 1.2	12.5	240 ± 33	30	200

[a] A weight of compound suspended in 0.2 ml of saline-Tween 80 was rated as active when it caused a significant ($P < 0.05$ reduction in the circumference of the tibiotarsal (ankle) joint compared with controls given a s.c injection of 0.2 ml of saline-Tween 80. The lowest active dose was multiplied by 8 to present the tabulated does in mg kg^{-1}.

[b] A weight of compound suspended in 0.2 ml of saline-Tween 80 was rated as active when it caused a significant decrease ($P < 0.05$) in the adjusted weight of granuloma tissue encapsulating the cotton pellets compared with controls receiving only 0.2 ml of saline-Tween 80. The lowest active dose was multiplied by 5 to present the tabulated dose in mg kg^{-1}.

[c] A weight of compound suspended in 0.2 ml of saline-Tween 80 was rated as active when it caused a significant decrease ($P < 0.05$) in the circumference of the tibiotarsal (ankle) joint compared with controls receiving 0.2 ml of saline-Tween 80. The lowest active dose was multiplied by 6 to present the tabulated dose in mg kg^{-1}. All doses were given by subcutaneous (s.c.) injection. A = active, I = inactive, NT = not tested, LD_{50} = lethal dose in 50% of the rats given a single s.c. injection and observed for at least 6 days, and TI = 'therapeutic index' (LD_{50}/ED_{95}).

related to the amount of Cu in them. Cupric acetate which was only effective in the CPE model contained nearly 32% Cu while Cu(II)(anthranilate)$_2$ and Cu(II)-(3,5-dips)$_2$ which were active in all three models of inflammation contained 18.9 and 12.5% Cu respectively. Therefore, to account for the observed anti-inflammatory activity it is necessary to invoke the existence of the intact complex since the observed activity cannot be attributed to the activity of anthranilic acid, 3,5-dips or the amount of Cu in the complexes.

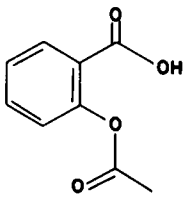
In addition, the rat LD_{50} data indicated that Cu(II)(anthranilate)$_2$ and Cu(II)-(3,5-dips)$_2$ were safe to use. This safety was quantitated by calculating the therapeutic index (TI), which is given in *Table 5.5* as the ratio of LD_{50}: ED_{95}, for these 2 compounds. Since an ideal TI is more than 10, the TI data obtained for these Cu complexes demonstrate that they are unusually safe to use.

These 2 Cu complexes were also evaluated with regard to their production of signs of central nervous system (CNS) toxicity. Subcutaneous injection of doses up to 320 mg kg^{-1} failed to produce signs of CNS stimulation or depression in mice. This was consistent with the lack of observed CNS effects in all of the anti-inflammatory testing of these two complexes in rats although Cu(II)$_2$(acetate)$_4$ had produced CNS toxicity of unknown pathogenesis at all active doses in rats. It was also found that mouse LD_{50} values for both of these compounds were greater than 320 mg kg^{-1}, the largest dose given.

To investigate possible liver damage in association with these 2 Cu complexes, 20 mg kg^{-1} (1 to 20 times the effective doses) were given for 10 days. The usual liver function regimen was 10 mg kg^{-1} for 4 days, so that giving twice as much for two and one-half times as long was not an experiment weighted in favour of the 2 complexes. In addition, 2 doses of Cu(II)$_2$(acetate)$_4$ 20 mg kg^{-1} and 10 mg kg^{-1} were tested in the same protocol to distinguish its toxicity from the 2 complexes. Neither Cu(II)(anthranilate)$_2$ nor Cu(II)(3,5-dips)$_2$ caused any liver function or liver cell damage, although Cu(II)$_2$(acetate)$_4$ caused some liver cell damage at both doses [37].

Having demonstrated an increase in anti-inflammatory activity for the Cu complexes of anthranilic acid and 3,5-dips as well as their safe use it was decided to test the hypothesis that Cu complexes of the clinically used anti-arthritic drugs might be more effective as anti-inflammatory agents than the parent drugs. For this purpose, Cu complexes were prepared using acetylsalicylic acid (aspirin) (12), 2-[3-(trifluoromethyl)phenyl]aminonicotinic acid (niflumic acid, 13) D-penicillamine (D-pen) (14), 1-phenyl-5-aminotetrazole (fenamole, 15) and salicylic acid (16).

Only the Cu complexes of aspirin, tetrakis-μ-acetylsalicylatodicopper(II) [Cu(II)$_2$(aspirinate)$_4$] (17) and salicylic acid (7) have been structurally characterized [87,88]. The binuclear structure of Cu(II)$_2$(aspirinate)$_4$, like that of Cu(II)$_2$-(acetate)$_4$ [tetrakis-μ-acetatodicopper(II)] and other carboxylic acid Cu



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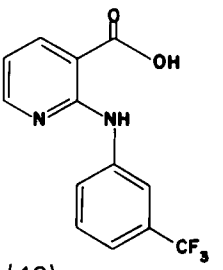

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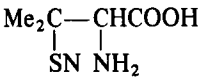

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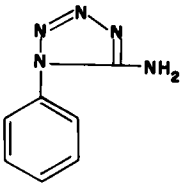

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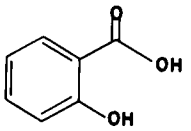

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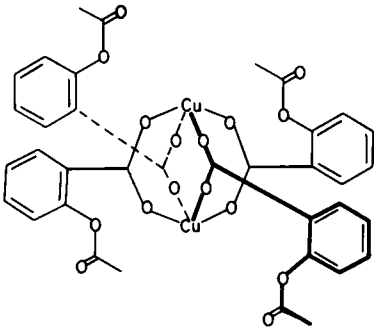


(17)

complexes are believed to result from intramolecular bonding contributions of the 4 carboxalate groups which facilitate spin coupling of the unpaired electron on each Cu atom by a super-exchange mechanism. A more detailed discussion of this octahedral bipyrimidal conformation had been presented elsewhere [37] along with plausible Cu complex structures of the remaining anti-arthritic drugs cited above.

The results of tests in the CPE, CWG and AA models of inflammation are presented in *Table 6.6.* Comparing the activity observed for aspirin and for its Cu complex demonstrated that $Cu(II)_2$(aspirinate)$_4$ was more active than aspirin.

Table 5.6. COMPARISON OF THE ANTI-INFLAMMATORY ACTIVITIES OF SOME ANTI-ARTHRITIC DRUGS AND THEIR Cu COMPLEXES [a] [37]

Compound	*Carrageenan paw oedema*	*Cotton wad granuloma*	*Adjuvant arthritis*	*%Cu*	LD_{50}	*TI*	
						CPE	*PA*
Aspirin	A at 64	A at 200 ig	A at 6		1500 ig 790 rt		
$Cu(II)_2$ (aspirinate)$_4$	A at 8	A at 10	A at 1.2	15.0	760 + 100	95	633
Niflumic acid	A at 40 ig	A at 25 ig	A at 6 ig		370 ± 25 ip	9	62
$Cu(II)_n$ (niflumate)$_{2n}$ $(H_2O)_n$	A at 8	A at 10	A at 1.2	10.1	650 ± 80	81	542
D-Penicillamine	I at 200	I at 100	I at 30				
Cu(I)D-pen$(H_2O)_{1.5}$	A at 8	A at 10	NT	26.7			
$Cu(II)_n$(D-pen)$_{2n}$ $(H_2O)_{2n}$	I at 200	NT	NT	16.1			
Cu(II)(D-pen disulfide)$(H_2O)_2$	A at 8	A at 25	A at 30	15.4			
Fenamole	I at 200	A at 100 ig	A at 100 ig				
$Cu(II)_n$(fenamole)$_n$(acetate)$_{2n}$	A at 8	A at 25	A at 30	18.5			
$Cu(II)_m$(fenamole)$_{2n}$$(HCl)_{2n}$	A at 16	A at 10	NT	13.9			
$Cu(II)_2$ (salicylate)$_4$ (Na_4)	A at 16	A at 10	NT	16.4			
Cu(II)(salicylate)$_2$ 4 H_2O	A at 8	A at 25	NT	15.4			

[a] See footnotes to *Table 5.5.* All compounds were given by s.c. injection unless indicated as intragastric (i.g.).

In the CPE model, the complex was active at 1/8 of the lowest active dose of aspirin. No s.c. data are available for aspirin in the CWG model so that comparison here is based upon oral data for aspirin. In the AA model, the anti-inflammatory activity of $Cu(II)_2(aspirinate)_4$ was found to be 5 times greater than the activity observed for aspirin. In addition, cupric aspirinate was active at 30 mg kg^{-1} when given intravenously while aspirin was inactive. Intravenous testing was carried out because it was suggested that a compound which was active because it was an irritant, may not be active when given intravenously.

The Cu chelate of niflumic acid was also found to be active in all three models. Comparison here is somewhat lacking, since one cannot directly compare activities of two compounds given by different routes of administration. However, the following comparison is viewed as helpful with regard to disclosure of potentially useful information. The parent compound was active at 40 and 25 mg kg^{-1} orally in the CPE and CWG models respectively, while the chelate was active at 8 and 10 mg kg^{-1} s.c. in these 2 models. The lowest active dose in the AA model was 6 mg kg^{-1} orally for niflumic acid and 1.2 mg kg^{-1} s.c. for $Cu(II)_n(niflumate)_{2n}$. When both $Cu(II)_n(niflumate)_{2n}$ and niflumic acid were tested intravenously in the adjuvant arthritic rat, the lowest active dose of niflumic acid was only 30 mg kg^{-1} while its Cu complex was active at 6 mg kg^{-1}.

Clinical use of D-penicillamine (D-pen) as an anti-arthritic compound is interesting since it is well-known that it is inactive in these animal models of inflammation at the large initial screening doses used in these three models of inflammation. However, two Cu coordination compounds obtained with D-pen, one a polymeric cuprous complex and the other considered to be a cupric disulfide complex, were found to be active in these models. The cupric bis-D-pen compound was found to be inactive at the initial screening dose in the CFE model and was not tested in the subsequent followup models of inflammation.

Activities of both coordination compounds prepared from fenamole were just as remarkable. The parent compound was inactive at the initial screening dose in the CPE model, active only at the initial screening dose in the CWG model and only active at 4 times the usual initial screening dose in the AA model of inflammation. However, the 2 fenamole Cu complexes were found to be active at low doses in the CPE and CWG models. In the AA model, only one of the 2 complexes was tested but it was found to be active. The 2 Cu salicylate complexes were also found to be potent anti-inflammatory agents in the CPE and CWG models of inflammation. These results are particularly significant in the light of Hangarter's successful therapeutic results with his preparation of Cu salicylate [67,68,71,72].

The anti-inflammatory activities of Cu complexes synthesized with inactive and active anti-inflammatory complexing agents along with similar results obtained with 12 other amino acid, carboxylic acid, amine and corticoid Cu

complexes [37] support the hypothesis that Cu coordination compounds which can be formed *in vivo* may account for the clinical usefulness of the anti-arthritic drugs. The suggestion that this beneficial activity is due to the complex in question is also supported by the lack of a direct correlation between anti-inflammatory activity and the amount of Cu in these compounds.

A comparison of the acute toxicity data obtained for some of these complexes and their parent compounds suggests that the Cu chelates are less toxic. Oral and rectal LD_{50} values for aspirin have been reported to be 1500 and 790 mg kg^{-1} respectively [89]. The LD_{50} value for $Cu(II)_2$(aspirinate)$_4$ was found to be 760 mg kg^{-1} when given s.c. The case concerning niflumic acid is more certain. The LD_{50} value for this chelate, 650 mg kg^{-1} s.c., was much higher than the value obtained for the parent compound given by the oral route, 370 mg kg^{-1}. The LD_{50} for the niflumic acid given intraperitoneally was found to be 155 ± 9 mg. It is possible that these Cu complexes may be more potent as anti-inflammatory agents and less toxic than their parent compounds.

In addition to this marked reduction in toxicity, some of these complexes were found to be potent *anti-ulcer* agents. Since it is well-known that clinically used anti-arthritic drugs cause ulcers and gastrointestinal distress, the observed anti-ulcer activity further distinguishes these coordination compounds from their parent compounds as being safer and potentially much more therapeutically useful than the currently used drugs.

Anti-ulcer activities of these complexes which are presented in *Table 5.7* were determined in the Shay ulcer model following *intragastric (IG)* or *oral* dosing.

Copper(II) acetate was found to be only very weakly active as an anti-ulcer

Table 5.7. ANTI-ULCER ACTIVITIES OF SOME Cu COMPLEXES [37]

Compound	*Shay anti-ulcer activity* [a]
$Cu(II)_2$ (acetate)$_4$ $(H_2O)_2$	A at 225
Cu(II)(anthranilate)$_2$	A at 4.5
Cu(II)(3,5-dips)$_2$	A at 2.3
$Cu(II)_2$ (aspirinate)$_4$	A at 11.3
$Cu(II)_2$ (niflumate)$_{2n}$	A at 4.5
$Cu(II)_2$ (D-pen)$_{2n}$	A at 4.5
Cu(II)(D-pen disulfide) · 3 H_2O	A at 4.5
$Cu(II)_n$ (fenamole)$_n$ (acetate)$_{2n}$	A at 4.5
$Cu(II)_n$ (fenamole)$_{2n}$ (HCl)$_{2n}$	A at 4.5
Cu(II)(salicylate)$_2$ · 4 H_2O	A at 4.5

[a] A weight of compound suspended in 1 ml of saline-Tween 80 was rated as active when it significantly inhibited ulceration ($P < 0.05$) compared with controls given only saline-Tween 80. The lowest active dose was multiplied by 4.5 to present the tabulated dose in mg kg^{-1}. All doses were given intragastrically.

compound. Activity at 225 mg kg^{-1} is viewed as a very low order of potency. However, Cu(II)(anthranilate)$_2$, Cu(II)(3,5-dips)$_2$, Cu(II)$_2$(aspirinate)$_4$, Cu(II)$_n$-(niflumate)$_{2n}$, Cu(II)$_n$(D-pen)$_{2n}$, Cu(II)(D-pen disulfide), Cu(II)$_n$(fenamole)$_n$-(acetate)$_{2n}$, Cu(II)$_n$(fenamole)$_{2n}$(HCl)$_{2n}$ and Cu(II)(salicylate)$_2 \cdot 4H_2O$ along with 27 amino acid, carboxylic acid, amine and corticoid-21-phosphate Cu complexes were found to be potent anti-ulcer compounds [37,38]. These results are particularily significant since the most common reason for withdrawal from aspirin and D-penicillamine therapy of arthritic diseases is the gastrointestinal irritation they cause. The data also support Hangarter's observation that patients treated with his preparation of Cu salicylate did not suffer from gastrointestinal distress.

In addition to the Shay anti-ulcer activity, a number of these chelates were also shown to have anti-ulcer activity in the corticoid-induced ulcer model [37]. Preliminary studies of Cu(II)$_{3n}$(HC-21-phosphate)$_{2n}$(H_2O)$_{9n}$ demonstrated that it inhibited ulcers in this model at doses as low as 18 mg kg^{-1}.

All of the Cu complexes that were active anti-ulcer compounds were also shown to decrease gastrointestinal secretions. Both acid and pepsin in these secretions were decreased, 5 hours after ligation, in the Shay rat. The possibility that these Cu complexes decreased pepsin activity by inhibiting pepsin was studied *in vitro* but no pepsin inhibition was found. The observed anti-secretory activity cannot be explained as anticholinergic activity since the Cu coordination compounds studied failed to posssess any significant ganglionic or post-ganglionic cholinergic blocking activity as shown in *Table 5.8.* This lack of anti-cholinergic activity or blockade of the autonomic nervous system was consistent with the observations that most of these compounds failed to affect psychomotor behaviour when treated with 6 doses of complex ranging from 5 to 320 mg kg^{-1}. Acute toxicity was not observed in this dose range when they were given s.c.

It was simultaneously suggested by Whitehouse, Field, Denko and Ryall [90] that D-penicillamine might be a precursor drug and the active species formed *in vivo* might be a Cu-D-penicillamine complex since a Cu complex of D-penicillamine was more active as an anti-inflammatory agent than D-penicillamine in the AA, CPE, urate paw edema (UPE) and oleyl alcohol paw oedema models of inflammation. These observations were extended by Whitehouse [91] in his discussion of the role of Cu in inflammatory disorders and with the report by Denko and Whitehouse [92] that Cu complexes of glycine and citric acid had anti-inflammatory activity in a model of calcium pyrophosphate-induced inflammation. Whitehouse and Walker [93] subsequently reported that Cu(II)-(D-Penicillamine)$_2$, Cu(I)-D-Penicillamine, Na_5Cu(I)$_8$Cu(II)$_6$-(D-Penicillamine)$_{12}$Cl, Na_2Cu(I)-thiomalate, Cu(I)-dithiodiglycol, $Na_2Cu_2(S_2O_2)_2 \cdot H_2O$, Cu(I)-(thioacetamide)$_4$Cl, Cu(I)($Cu_3CN$)$_4CO_4$, Cu(I)Cl · dimethylsulfoxide, bisglycinatocopper(II) and Cu(II)-ascorbate were effective in CPE, UPE, kaolin paw oedema

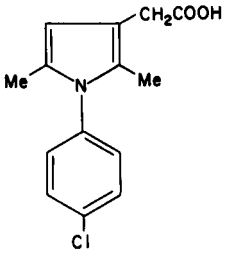
Table 5.8. ANTICHOLINERGIC ACTIVITY, QUALITATIVE MOUSE BEHAVIOURAL EFFECTS AND ESTIMATIONS OF LD_{50} VALUES IN MICE FOR SOME Cu COMPLEXES [37]

Compound	*Anticholinergic activity* [a]		*Qualitative behavioural change (mg kg^{-1})* [c]	*LD_{50} (mg kg^{-1})* [c]
	% TEA [b]	*% atropine*		
$Cu(II)(anthranilate)_2$	<11	<1	I at 320	>320
$Cu(II)(3,5\text{-dips})_2$	<20	<1	I at 320	>320
$Cu(II)_2(aspirinate)_4$	<20	<1	I at 320	>320
$Cu(II)_n(niflumate)_{2n}(H_2O)_n$	NT	NT	I at 320	>80 < 320
$Cu(I)_n$ D-pen$(H_2O)_{1.5n}$	NT	NT	I at 320	>80 < 320
$Cu(II)_n(D\text{-pen})_{2n}(H_2O)_{2n}$	NT	NT	Depressant at 320	>80 < 320
$Cu(II)_n$(D-pen disulfide)$_n(H_2O)_{3n}$	NT	NT	I at 320	>80 < 320
$Cu(II)_n(fenamole)_n(acetate)_{2n}$	<7	NT	I at 320	>320
$Cu(II)_n(fenamole)_{2n}(HCl)_{2n}$	<7	NT	I at 320	>320
$Cu(II)_2(salicylate)_4(Na)_4$	NT	NT	I at 320	>320
$Cu(II)(salicylate)_2(H_2O)_4$	<20	<1	I at 320	>320

[a] Less than (<) values indicate no difference from control values.
[b] Tetraethylammonium bromide.
[c] All doses given s.c.

and/or AA models of inflammation following s.c. dosing. A comparison of Cu, gold (Au) and silver (Ag) thiomalate and thiosulphate complexes in these models of inflammation revealed that the Cu complexes were effective in preventing inflammation and compared favourably in this respect with proven anti-inflammatory drugs while the Au and Ag complexes were virtually inactive, even at higher doses. These data support the results obtained by Forestier in his clinical comparisons of Cu and Au complexes.

Following the report [86] that $Cu(II)_2(aspirinate)_4$ had more anti-inflammatory activity than aspirin in the CPE and AA models of inflammation when they were given s.c., Williams, Walz and Foye [94] reported that $Cu(II)_2$-$(aspirinate)_4$ was only as active as aspirin in the CPE inflammation but about twice as active as aspirin in AA when they were given orally. Rainsford and Whitehouse [95] also found that $Cu(II)_2(aspirinate)_4$ and a preparation of Cu(II)-salicylate had the same activity as aspirin and salicylic acid in CPE and UPE when they were given orally. Boyle, Freeman, Goudie, Magan and Thomson later reported [96] that Cu complexes of aspirin (12), clopirac (198), ketoprofen (19), (+)-naproxen (20), niflumic acid (13) and indomethacin (21) were only as active as the parent drugs when given orally in CPE.



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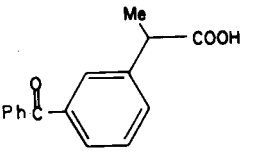

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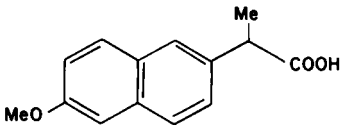

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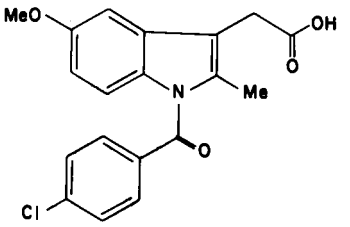

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It has been suggested [97] that the reasons for the reduced oral anti-inflam-

matory activity found by Williams, Walz and Foye [94] and Rainsford and Whitehouse [95] were in part gastric acid destruction and the use of ionic suspending agents, tragacanth and acacia, which are capable of either removing Cu from these complexes or forming quaternary complexes. If Cu were removed from the complex, then only the parent drug would be available for absorption. If a quaternary complex were formed, it is likely that only a small amount of complex would be absorbed. However, methylcellulose was used as the suspending agent in the Boyle, Freeman, Goudie, Magan and Thomson [96] study, so it appears that gastric acidity may be the primary cause for the reduced activity found following oral dosing of these animals. Nevertheless, ionic suspending agents such as tragacanth, acacia and carboxymethylcellulose should always be avoided since they may affect the results when gastric acidity is of no concern. A third reason for reduced anti-inflammatory activity of these Cu complexes following oral dosing may have been a decreased rate of absorption in the stomach. When there is a slower rate of absorption and the complexes pass into the small intestine, they are no longer likely to be absorbed since Cu complexes split in basic media and yield copper(II) oxide. When this occurs neither the complex nor the Cu is available for absorption since the oxide is very insoluble in basic media.

When $Cu(II)_2(aspirinate)_4$ and Cu(II) salicylate were given by s.c. injection, Rainsford and Whitehouse [95] found that they were 5 to 6 times more active against CPE and UPE than when tested by the oral route of administration and provided additional support for the results obtained by Hangarter [68,71,72]. Boyle, Freeman, Goudie, Magan and Thomson [96] also confirmed the observation that $Cu(II)_2(acetate)_4$ was more active in the CPE model when given s.c. than orally. This increased activity following s.c. administration may be an irritant-induced anti-inflammatory effect [95]. However, reported data [37] which were in part obtained in consideration of that possibility do not support that suggestion.

$Cu(II)_2(acetate)_4$ which caused marked irritation on s.c. injection was only active in the CPE model of inflammation and had no anti-inflammatory effect in the CWG and AA models of inflammation, even though it was just as irritating in the animals used in these studies [37]. In contrast to this experience, many Cu complexes were found to be active in all 3 models of inflammation following s.c. administration and were not noted to cause irritation. For example, a large dose of $Cu(II)(tryptophan)_2$, (200 mg kg^{-1}) caused some irritation at the site of injection but it was inactive s.c. as an anti-inflammatory agent in the CPE model. Although it was active orally in this model and lacked oral irritating properties, it had anti-ulcer activity at a dose as high as 225 mg kg^{-1}. The rats used in the CWG model of inflammation were adrenalectomized, and compounds which were active because they were irritants and stimulated adrenal production of the

corticoids would not be active in this model. Yet, all of the Cu complexes studied were shown to be active. It was also suggested that compounds which caused irritation at the sight of injection and as a result were anti-inflammatory would be inactive when given i.v. The Cu complexes given i.v. were also active in in the AA model of inflammation although some of the parent clinically used drugs were inactive. Irritation resulting in stimulation or depression of the central nervous system was also suggested as a mechanism of irritant-induced anti-inflammatory activity. However, studies with large doses of Cu complexes failed to reveal any central nervous system stimulation or depression. As an alternative to the toxic-irritant mechanism of action, the increased anti-inflammatory activity of the Cu complexes given s.c. may be the result of achieving greater concentrations of the active species in the inflamed tissues. This active species may facilitate physiological processes required for the return to normal tissue status.

Some of the more recent studies of Cu complexes have been carried out with large doses to evaluate their gastric irritation or ulcerogenic properties since all of the currently used anti-arthritic drugs are known to cause gastrointestinal distress and/or ulcers. Williams, Walz and Foye [94] were the first to report that $Cu(II)_2(aspirinate)_4$ caused no gastrointestinal erosions in rats with doses up to 1380 mg kg^{-1} of the complex whereas aspirin produced a 50% or greater incidence of erosions at much lower doses of 100–300 mg kg^{-1}. Rainsford and Whitehouse [95] found that their preparation of Cu(II) salicylate and $Cu(II)_2$-$(aspirinate)_4$ either reduced or prevented gastric damage in Rainsford's cold-stress rat ulcer model. Even a large dose of Cu(II) salicylate caused no gastric damage in rats with AA. The mechanism of this protective effect was suggested to be a stimulation of gastric mucous effusion by Cu(II) ions [95,98]. However, since it is unlikely that the ionic species of Cu(II) exists in any significant concentration in this biological system, the lack of significant anti-ulcer activity for $Cu(II)_2(acetate)_4$ [37] which is readily dissociable, suggests that the existence of Cu complexes must be invoked to account for the variation in anti-ulcer activity following treatment with various complexes. It has also been suggested that the anti-ulcer activity observed by Rainsford and Whitehouse [95,98] might have been greater had they not first acidified the Cu solutions before administration as this is likely to at least partially dissociate these metal complexes [97]. Boyle, Freeman, Goudie, Magan and Thomson [96] found that the Cu complexes of ketoprofen (19), (+)-naproxen (20) and indomethacin (21) were as ulcerogenic as their parent compounds but the Cu complexes of aspirin (12), niflumic acid (13) and clopirac (18) failed to produce the gastric damage caused by the parent drugs. The ulcerogenic Cu complexes were shown to be equally effective as their parent compounds in inhibiting 'total' prostaglandin synthesis but the non-ulcerogenic Cu complexes failed to cause the inhibition of 'total' prostaglandin

synthesis observed for the parent drugs. These effects on prostaglandin synthesis were suggested to account for the observed ulcerogenicity. $Cu(II)_2$(aspirinate)$_4$ was studied with regard to its effect on PGE and $PGF_{2\alpha}$ synthesis and it was found that this complex caused a concommitant increase in $F_{2\alpha}$ and decrease in E synthesis. Although the relative changes in synthesis of these prostaglandins are consistent with data in the literature (see following Section), the magnitude of these changes is somewhat in doubt since the *in vitro* test system used in these studies contained EDTA which may complete for the Cu in these complexes and the tests were done at pH 8.0, at pH at which insoluble polynuclear coordination complexes are formed causing the Cu to be less available for biochemical reaction process(es).

The question has been raised concerning toxicity associated with the use of a Cu-containing substance in therapy even though they were found to be less toxic than the parent compounds including the clinically used drugs [37], lacked gastrointestinal irritation [94,96] and possessed anti-ulcer activity [37,95,98]. From this evidence, it is possible that rheumatoid diseases may require Cu in a complexed form to counteract pathogenic processes associated with these diseases so that remission can be achieved. In this regard, if a Cu complex is less toxic and more effective than existing drugs, then the fact that the complex contains Cu should be viewed positively.

BIOCHEMISTRY OF ANTI-INFLAMMATORY COPPER COMPLEXES

During the last few years, important gains have been made concerning possible biochemical mechanisms of action for these Cu complexes. There are at least 5 plausible biochemical mechanisms that can be invoked to account for their anti-inflammatory and anti-ulcer activities. These are: induction of lysyl oxidase, modulation of prostaglandin syntheses, induction of superoxide dismutase or superoxide dismutase mimetic activity, stabilization of lysosomal membranes. and modulation of the activity of histamine.

Since it is well known that repair of sites of inflammation, including ulcers, requires the cross-linking and extracellular maturation of the connective tissue components, collagen and elastin, and that the enzyme responsible for this, lysyl oxidase, is a Cu-dependent enzyme [99,100], this aspect of wound or tissue repair assumes particular significance with regard to a role for Cu complexes having both anti-inflammatory and anti-ulcer activity. Recently, Harris has demonstrated that lysyl oxidase activity is induced in Cu-deficient chickens with copper(II) sulphate [101]. He has also been able to induce lysyl oxidase activity in these animals with Cu(II)(anthranilate)$_2$, Cu(II)(3,5-dips)$_2$ and $Cu(II)_2$-(aspirinate)$_4$ [101a].

In the light of these observations, the absence of ulcerogenicity [94] and the observation of anti-ulcer activity [37,38,95] with these and other Cu complexes merits special consideration. If one possible chemical mechanism for ulcerogenesis is the loss of Cu from the Cu-dependent enzyme lysyl oxidase, which is required to maintain the integrity of the extracellular connective tissue in the mucosa and submocosa, then the ulcerogenicity of aspirin and other anti-inflammatory compounds may be due to their ability to form Cu complexes resulting in enzyme loss as a direct consequence of their coordination chemical reactivity. It follows then that existing ulcers can be successfully treated with Cu complexes which can induce lysyl oxidase activity and promote tissue repair.

The suggestion that these compounds promote tissue repair is consistent with the observation that Cu(II)(L-tryptophan)$_2$-treated rats, with surgically-induced ulcers (or wounds) [102] appeared to heal at a markedly increased rate compared with nontreated controls. Townsend found that at day 5, post-ulcer induction treated animals which were given 25 mg kg^{-1} Cu(II)(L-tryptophan)$_2$ beginning on day 1 post-surgery, were a full 5 days ahead of the non-treated control animals with regard to healing of these ulcers, and they remained ahead of the non-treated controls throughout the course of the 20-day experiment. In this same ulcer model, 25 mg kg^{-1} of Cu(II)$_2$(aspirinate)$_4$ also brought about the same increase in rate of ulcer repair [103]. In addition, visual comparison of non-treated and Cu(II)(L-tryptophan)$_2$ or Cu(II)$_2$(aspirinate)$_4$-treated rats revealed that adhesions had formed only in non-treated rats.

Histochemical studies of the Cu(II)$_2$(aspirinate)$_4$-treated rats revealed that regression adjacent to the lesion seen at 5 days post-surgery in non-treated rats was absent in treated rats and the size of the ulcer or wound was much smaller in treated rats as a result of increased re-epethelialization and gland formation [103]. On day 10 post-surgery, re-epithelialization was completed in only one of the non-treated rats, new glands were highly irregular and the muco-collagenous band joining the cut ends of the muscularis mucosae was discontinous and fenestrated. In contrast, re-epithelialization was complete in all treated rats, new glands were regularly shaped, and the muco-collagenous band was continuous and thicker. In the non-treated rats, collagen in newly formed mucosa and submucosa at the base of the lesion was very dense and composed of thick wavy disorientated bundles, while treated rats had normally orientated fine collagen fibres in the same location. The quantity and quality of the replaced collagen in the treated animals were superior to that found in the non-treated animals. While the desired collagenous changes were interpreted as being the result of lysyl oxidase induction, the earlier anti-inflammatory effects were suggested to be the result of Cu(II)$_2$(aspirinate)$_4$ modulation of prostaglandin synthesis [103].

Modulation of prostaglandin synthesis, by Cu complexes may result in a decrease in the synthesis of the pro-inflammatory (vasodilator) prostaglandin,

PGE_2, and a concomitant increase in the synthesis of the anti-inflammatory prostaglandin (vasoconstrictor), $PGF_{2\alpha}$ [86]. This suggestion is consistent with the report of Lee and Lands [104] which has been confirmed by Maddox [105] who found a depression in PGE_2 synthesis and an increase in $PGF_{2\alpha}$ following the addition of $CuSO_4$ [104] or $CuCl_2$ [105] to seminal vesicle homogenates. Additional support for these observations has recently been provided by Vargaftig, Tranier and Chignard [106] who found Cu to be required for platelet synthesis of prostaglandins from arachidonic acid and suggested that this accounted for the observed anti-inflammatory activity of Cu complexes. This suggestion has been recently supported by Boyle, Freeman, Goudie, Magan and Thomson [96] who demonstrated that $Cu(II)_2(aspirinate)_4$ brought about a concommitant decrease in PGE and increase in $PGF_{2\alpha}$ synthesis. They also confirmed the results of Lee and Lands [104] and Maddox [105] by showing that $CuCl_2$ caused a decrease in the synthesis of PGE while increasing the synthesis of $PGF_{2\alpha}$.

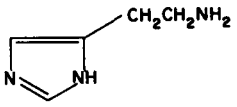
Rheumatoid arthritis may be the result of a deficiency or lack of superoxide dismutase enzyme activity [107]. This suggestion is of interest, because it is known that human superoxide dismutase contains Cu, which is required for its dismutase activity [108]. In addition, it has been reported [109–111] that the Cu chelates: $Cu(II)(lysine)_2$, $Cu(II)(glycylhistidine)_2$, $Cu(II)$-(glycylhistidyl-leucine)$_2$, $Cu(II)(tryptophan)_2$, and $Cu(II)(histidine)_2$ have superoxide dismutase activity. Since it had been shown that $Cu(II)(tryptophan)_2$ and $Cu(II)(lysine)_2$ had anti-inflammatory and anti-ulcer activity, other anti-inflammatory Cu chelates may have superoxide dismutase activity [38]. This suggestion has recently been supported with the report by deAlvare, Goda and Kimura [112] that $Cu(II)(salicylate)_2 \cdot 4H_2O$, $Cu(II)_2(aspirinate)_4$ and $Cu(II)_2(niflumate)_4$ have superoxide dismutase activity.

The superoxide dismutase mimetic activity observed for $Cu(II)(tryptophan)_2$ and $Cu(II)_2(aspirinate)_4$ may account for the observation that mucosal and submucosal tissues of surgically treated rats with these 2 complexes did not undergo autolytic destruction along the borders of the original incision as they did in the non-treated control animals. This dismutase activity may also account for the absence of hard liver, spleen, pancreas and stomach adhesions seen only in the non-treated controls [103].

A fourth possible biochemical mechanism of action for Cu complexes is based upon the report of Chayen, Bitensky, Butcher and Poulter [34] that Cu was important for redox control of human synovial lysosomes. They found that Cu decreased the permeability of lysosomes and, in addition, decreased the ratio of free versus bound lysosomal enzymes. This is an alternative biochemical mechanism which may be used to account for the absence of autolytic destruction and adhesions associated with the use of Cu complexes in the treatment of surgically-

induced ulcers [103]. The possibility that the anti-inflammatory activity of the Cu complexes was due to lysosomal enzyme inhibition has been partially ruled out by McAdoo, Dannenberg, Hayes, James and Sanner [113] who found that many complexes failed to inhibit cathepsin-D, a lysosomal proteinase.

Modulation of the physiological effects of histamine (Hm) (22) may also be an important biochemical role for Cu complexes. Walker, Shaw and Li [114], Walker, Reeves and Kay [115], and Walker and Reeves [116] considered the coordination chemistry of histamine *in vivo* and provided an abundance of evidence to support the suggestion that a binuclear, hydroxy-bridged Cu complex (23) was the active form of histamine. Intraperitoneal injections of



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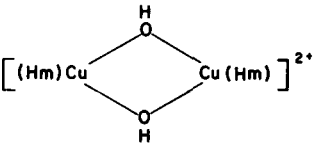

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Cu(II)(histamine)Cl_2 at 1/50th the dose of histamine produced the same subliminal anaphylactic symptoms as histamine. Injection of $CuSO_4$ 2 min before the injection of a non-lethal dose of histamine caused death by anaphylactic shock in all mice within 10 min. The anaphylactic LD_{50} value for Cu(II)(histamine)Cl_2 was found to be 1/16th the anaphylactic LD_{50} dose of histamine. These data support the suggestion that histamine-induced vascular permeability as an acute phase inflammatory reaction may be mediated by a Cu-histamine complex following degranulation of mast cells in the connective tissue spaces.

With the recognition that a Cu complex of histamine was responsible for its histaminic activity, Walker and Reeves [116] suggested that a competition for this Cu accounted for the well-known observation that salicylic acid has anti-histamine activity. They then demonstrated that salicylic acid prevented lethality in mice given an LD_{50} dose of histamine. Since an increase in vascular permeability is an important physiological response in inflamed tissues, these observations merit in-depth study.

SUMMARY

Copper complexes are a unique class of anti-arthritic drugs for 2 important reasons. First, animal studies have revealed that they are potent anti-ulcer agents

in contrast to currently used ulcerogenic drugs. This is consistent with the lack of gastrointestinal irritation reported by Hangarter. Second, treatment of human rheumatoid diseases brought about long-lasting remissions with short term Cu complex therapy in contrast to chronic therapy with existing drugs. These 2 unique features of the biological activity of Cu complexes along with their demonstrated anti-inflammatory activity in many animal models of inflammation suggest that Cu complex therapy may be more beneficial than existing drug therapy. Copper complex therapy is also consistent with what has been interpreted as the normal physiological response to rheumatoid diseases.

Serum from patients with rheumatoid disease has been shown to contain increased concentrations of ceruloplasmin and low molecular weight Cu complexes. Since these increases occur prior to remission and low molecular weight Cu complexes are known to have anti-inflammatory activity, it is suggested that the increase in serum Cu containing components is a physiological response to these diseases. This physiological response then facilitates biological processes required to prevent further tissue destruction and promote tissue repair which leads to remission.

Recent pharmacological and biochemical studies have provided evidence for five plausible Cu-dependent mechanisms of action which may account for the observed biologic activity of Cu complexes. These are: induction of lysyl oxidase or lysyl oxidase mimetic activity, induction of superoxide dismutase or superoxide dismutase mimetic activity, modulation of prostaglandin synthesis, modulation of histamine activity, and lysosomal membrane stabilization. Ongoing research may provide evidence for still other Cu-dependent processes which may more fully explain the biologic activity of Cu complexes.

The possibility that rheumatoid diseases are associated with deficits in Cu-dependent processes also suggests that the etiology and/or chronicity of these diseases may result from a quasi Cu deficiency or the lack of low molecular weight complex forms of Cu.

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6 Non-Tricyclic Antidepressants

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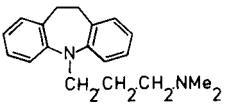
INTRODUCTION

Almost 2 decades ago the antidepressive properties of imipramine (1), a tricyclic compound, were noticed by Kuhn during clinical investigations: (1) gave no



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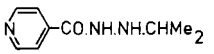

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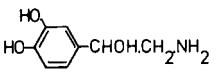
relief in psychotic patients, but Kuhn noted that it was most useful in various forms of depression [1].

Another pharmacotherapy of depression was developed in 1952 and was based on the observation that iproniazid (2), used as tuberculostatic, showed a favourable effect in depressed patients [2], believed to be based on monoamine oxidase (MAO) inhibition [3].

Today imipramine (1) is still one of the most commonly used antidepressants and a large number of structural analogues have been developed. Most of these tricyclic antidepressants show a closely related pharmacological profile, which may be a consequence of their resemblance in structure.

Concerning the mechanism of action, the tricyclic antidepressants possess an inhibiting effect on the presynaptical uptake mechanism of biogenic mono-



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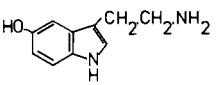

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amines, especially noradrenaline (NA) (3) and 5-hydroxytryptamine (5-HT) (4); this may be the basis of their therapeutic effect in depression [4].

Other actions associated with the tricyclics, e.g. their cardiovascular and their anticholinergic properties [5,6], must nowadays be considered as serious side-effects. Moreover a disadvantage of these tricyclics is the slow onset of action in patients (about 2 wk), whereas only 60–70% of those with endogenous depression respond to the therapy. In the use of MAO inhibitors as a therapeutic in depressed patients, the inhibition of this important enzyme system imposes limitations on their applications because of undesired interactions with other drugs and some foodstuffs. For that reason, much effort has gone into the finding of novel antidepressant structures, differing in pharmacological profile and aiming at the following improvements: increased activity, a more rapid onset of action and a diminished toxicity. Although an inhibition of the re-uptake of

both NA and 5-HT is still considered as an important feature, selectivity in influencing individual biogenic amines (especially NA and 5-HT) has become an important goal also. This resulted in the development and the clinical investigation of a large number of compounds with structures differing considerably from those of the tricyclics.

In the present review of recent developments in those so-called 'non-tricyclic antidepressants', the test methods on which the conclusion of 'probably therapeutically active' is based are evaluated. Then the activities of the various new structures, classified by structural resemblance, are discussed. Finally an attempt is made to derive some general structure-activity relations (SAR) and to draw some final conclusions about the whole subject. An index of compound names has been added as an aid to finding information about individual compounds and comparing the phases of development of these.

TEST METHODS

GENERAL CONSIDERATIONS

In general the therapeutic properties of tricyclic antidepressant drugs are nowadays attributed to their inhibition of a transmitter uptake by monoaminergic neurons in the central nervous system. This hypothesis is based mainly on the pharmacological properties of these tricyclics in animal experiments viz. the antagonism of monoamine depleting agents, the potentiation of directly or indirectly acting amine agonists, and the decrease in amine uptake processes by appropriate cells or cell fragments. This line of thought has in its turn led to the use of these aspects of the pharmacological profile for the identification of antidepressants among new chemical entities.

Such tests, however, suffer from major shortcomings:

(1) they are designed to detect acute effects of drugs, yet tricyclic antidepressants have a delayed onset of action in man; a desirable rapid onset of antidepressant action in humans is difficult to estimate in animals;

(2) in general, most tests identify potentiation of aminergic functions only when this is brought about in an identical way, as done by tricyclics, whereas other potentiation effects remain undetected;

(3) essentially other possibilities to restore the depressed patient remain undetected.

Especially this latter disadvantage gave rise to the search for other screening methods, in a number of cases aimed at the induction of a 'depressive state' in animals [7]. The realisation of such animal models, however, is laborious and their superiority to the commonly used techniques has still to be presented. The

majority of the medicinal chemical investigations therefore still rely on the resemblance of the pharmacological properties of the investigated series to those of the antidepressant tricyclics.

Investigations along these lines are then confronted with another problem, namely that the relative activity, in comparison with a standard compound such as imipramine (1), may differ essentially in the various animal models aimed at detecting one and the same property, such as noradrenaline re-uptake. Additional pharmacological properties of the studied compound may affect the various test models in different manners. Also basic properties of the test systems, e.g. the turnover rate of the biogenic amine involved in various strains of animals, may determine the results critically. These latter circumstances may be the cause of different results being obtained in different laboratories. For that reason, a short critical appraisal of the most commonly used methods seems appropriate.

RESERPINE ANTAGONISM

Most frequently used in these antagonism tests are reserpine and the benzoquinolizine derivatives, tetrabenazine and Ro-4-1284. The latter 2 drugs have a more rapid onset of action and a more selective activity on the central nervous systems (CNS) than reserpine. Antagonism by test compounds of the induced hypothermia, ptosis (eyelid closure), miosis (decreased pupil diameter), and sedation is measured either separately or combined. Evidence has been presented [8] that hypothermia antagonism is a better indicator of CNS activity, as ptosis and miosis can be influenced also by drugs with a peripheral action [9].

An extensive study with a large number of compounds in the Ro-4-1284 test [10] revealed that many currently used drugs produced antagonism of the Ro-4-1284 induced symptoms, thus pointing to the possibility of many 'false positive' results. The most important groups of compounds included in the study were MAO inhibitors (as most pronounced antagonists), sympathomimetics (ptosis counteraction), antihistamine drugs, and amphetamine-like compounds. For the detection of imipramine-like drugs, all these 'false positive' possibilities have to be excluded by further screening procedures. Special attention must be paid to differences in properties of psychostimulants and antidepressants, e.g. amphetamine versus imipramine (1). Although amphetamines have been tested clinically in depressive states, they have not been found to be of therapeutic value [11]. Psychostimulants are pharmacologically differentiated by their strong stimulatory action on locomotor behaviour in intact animals, whereas antidepressants are not active in this situation.

Unless otherwise stated, mice are mostly used as test animals, and effective

dosages of tricyclic antidepressants are commenly in the range of 0.5–10 mg kg^{-1} i.p. [12].

DOPA-POTENTIATION

After partial inhibition of MAO (with pargyline 4–24 h prior to the test), the response to 3,4-dihydroxyphenylalanine (DOPA), e.g. squeaking, jumping and fighting, is markedly enhanced by tricyclic antidepressants. MAO inhibitors, of course, produce the same response without pretreatment with pargyline. A survey of possible interference with other classes of drugs has not been published.

INTERACTION WITH 5-HTP

In the reserpine antagonism and DOPA potentiation tests, responses are most probably caused by potentiation of NA-effects. In order to study the effects on 5-HT systems, 2 methods are being used: interaction with 5-hydroxytryptophan (5-HTP)-induced head-twitches in mice and potentiation of 5-HTP-induced gross behaviour in mice and rats.

MAO inhibitors potentiate, and imipramine antagonizes, head-twitches after 5-HTP. However, this antagonism is not selective and is also produced by 5-HT antagonists, antihistamines, major tranquillizers, and sympathicomimetic drugs [13].

Hyperactivity in rats, after a combination of L-tryptophan and tranylcypromine, is strongly potentiated by selective 5-HT-potentiating compounds [14]. A behavioural syndrome, consisting of repetitive head movements, treading with the forepaws ('piano playing'), lordosis, tremor and hyperthermia, in mice after 5-HTP can be used for the same purpose [15]. These behavioural elements are mediated not only by 5-HT but also via dopaminergic pathways.

A number of drugs with central effects can influence this 'serotoninergic' behaviour, so that, for example, the 5-HT uptake inhibiting properties will not become apparent when the compound has in addition some neuroleptic properties.

AMPHETAMINE POTENTIATION

A number of behavioural effects of amphetamine are potentiated by tricyclic antidepressants [16]. Motor activity induced in mice by amphetamine, as measured in activity cages (light beam crossing), is often used. Also, the reduction of

food consumption (rats) by amphetamine is potentiated by established antidepressants. These effects are thought to be mediated by an effect on the noradrenergic transmission.

Care must taken, however, with compounds which interfere with the metabolism of amphetamine. For instance, compound SKF 525A, an inhibitor of detoxification by the liver and lacking antidepressant properties, was found to be active. It has also been shown that imipramine inhibits the hydroxylation of amphetamine in the liver [17], a fact which complicates the interpretation of these test results.

IN VITRO EXPERIMENTS

The amine uptake inhibiting properties of potential antidepressant compounds can also be measured biochemically by means of isolated synaptosomes, artificially torn-off nerve endings which retain their transmitter uptake system [18]. Synaptosomes can be isolated from whole brain homogenates or brain areas. By incubation of these synaptosomes with radioactively labelled NA or 5-HT after preincubation with various concentrations of inhibiting compounds, the uptake-inhibiting properties of these compounds can be measured.

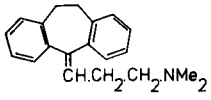
The disadvantage of these *in vitro* methods is the complete absence of any information about possible pharmacokinetic influences on the drug's action. This, however, can be overcome by administration of compounds to animals, mostly rats, and the isolation of synaptosomes from the brain of sacrificed animals after an appropriate interval. This is usually called the *'in vivo/in vitro'* method.

Instead of synaptosomes, blood platelets can also be used for uptake inhibition studies. Platelets contain an uptake mechanism for 5-HT and for NA which has properties analogous to that of nerve endings. This process can be studied effectively *'in vitro'* and it has been shown that tricyclic antidepressants inhibit the uptake of 5-HT and NA by platelets [19]. Measurements of the inhibition of uptake of monoamines by platelets, obtained from patients under treatment can provide an indication of the uptake inhibiting effectiveness of compounds in clinical trials.

Differences in technical details in the performance of this test can lead to important differences in the concentrations of standard compounds at which uptake inhibition occurs. For that reason, an actual comparison of the results of different laboratories is difficult, but selectivity of a compound towards NA or 5-HT uptake systems can be compared.

Apart from studies of uptake mechanisms, transmitter levels or turnover parameters (in rat or mouse brains) are also used as indicative information. In

accordance with the theory of uptake inhibition, alterations in steady state concentrations of 5-HT and/or NA are not to be expected, unless compounds possess MAO-inhibitory properties. In turnover studies, a decreased turnover as a result of an enhanced receptor activation can be shown for imipramine (1) and amitriptyline (5) [20]. The increase of NA-turnover shown by some of the non-



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tricyclic antidepressants, does not fit this conceptual mechanism of action. An increased turnover of brain NA is found, however, after subacute (3 days) or chronic (3 wk) treatment with tricyclic antidepressants [21,22].

CARDIOVASCULAR STUDIES

An inhibition of the uptake of NA in nerve endings of the sympathetic innervated blood vessels by tricyclic antidepressants can be demonstrated accurately with blood pressure measurements. Potentiation of the NA pressure response and antagonism of the effects of indirectly acting sympathomimetic amines, such as tyramine, are characterized by a rapid onset of action [23]. With proper precautions, these effects can also be studied in man. It must be remembered that these actions are on the peripheral nervous system and provide no guarantee of CNS-activity.

ANTICHOLINERGIC EFFECTS

As the most commonly occurring clinical side effects (dry mouth, cardiac toxicity) of tricyclic antidepressants are due to their anticholinergic (atropine-like) effects, it has become a major goal to eliminate this activity. To test anticholinergic activity, compounds are mostly investigated in respect of their spasmolytic activity against the cholinomimetic carbachol on the isolated guinea pig ileum. Also, the antagonism of cholinergic drug effects (tremorine, oxotremorine, pilocarpine) is used. Symptoms are, for example, salivation, lacrimation (peripheral), and tremor and piloerection (central effects). By means of a scoring system, effective antagonism of test compounds can be established.

MISCELLANEOUS

The search for antidepressant test methods, unrelated to the monoamine hypothesis, has become more and more important. In order to find new antidepressant mechanisms of action, new experiments are being devised which either demonstrate the effectiveness of known antidepressants or suggests a relation to the human depressed state.

Instinctive mouse-killing behaviour (muricide) in rats is blocked by tricyclic antidepressants, some antihistamines and amphetamine-like drugs [24]. This effect has been attributed to a selective inhibitory action on the amygdaloid area of the brain [25], an area which is involved in predatory behaviour. As this test method is not selective for antidepressants, all claims in favour of involvements of the amygdala in depression remain speculative.

The observation that the autonomic and psychic effects of yohimbine were potentiated in patients receiving imipramine [26] led to the use of yohimbine effects in mice to detect antidepressants [27]. An increased toxicity of yohimbine is indeed shown with imipramine but is not specific for antidepressants as such.

A better resemblance to the human depressive state in comparison with monoamine-depleted animals is claimed for animal separation models. Dog puppies separated from their mother display behavioural syndromes similar to human depression in which vocalization is most pronounced. Among a large number of drugs, imipramine was found most effective in reducing vocalization, without causing sedation [28].

Another behavioural syndrome, called 'learned helplessness' can be induced in dogs by presenting an inescapable traumatic event, e.g. electric shock in a shuttle box. When later confronted with a signalled escape-avoidance situation, two thirds of the animals develop a typical passive state and become 'helpless'. The development of helplessness in rats has been correlated with a depletion of brain NA, and when atropine is injected directly into the septal brain area of cats, their previously induced helplessness is cured [29]. Recently, a new animal model useful for screening potential antidepressive compounds has been described [30]. When rats are forced to swim in an inescapable situation, they will stop swimming and develop an immobility. The duration of this state of immobility can be shortened by treatment with antidepressants.

Finally the development of EEG studies, both in animals and in human volunteers, has to be mentioned. It is to be expected that computerized EEG analysis will provide means to classify and to detect psychotropic drug activity in man [31].

CLINICAL TRIALS

After proper toxicity studies in animals and tolerance studies in human volunteers, the clinical investigation in depressive patients of potential new antidepressants are in most cases started with so-called 'open label trials'. In these trials the observers know which drug has been administered to the patient. If the results seem promising, compounds are tested further in double-blind trials, conducted with a standard antidepressant as reference compound, e.g. imipramine (1) or amitriptyline (5), and/or with a placebo. During double-blind studies with a reference compound the two drug-periods are compared with a drug-free period with regard to a number of clinical parameters, mostly in the form of rating scales.

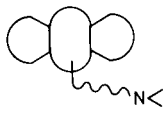
As in the treatment of depression the effect of mere hospitalization and also of a placebo can be very great, it often happens that compounds which seem valuable in the first open trials may, in a later double-blind trial, not show any significant difference from the effects of established drugs or even in comparison with placebo treated groups. This illustrates the limited value of the open trials.

Most studies concern patients with so-called endogenous depression. Trials with patients with various types of depression in one group may also impose limitations on the value of the results.

Clinical studies in psychiatry are often hampered, too, by different diagnostic criteria. Efforts in biochemical differentiation [32] between NA- and 5-HT metabolism in depressive patients are under way [33], but the value of this differentiation has still to be proved.

SELECTION AND CLASSIFICATION OF COMPOUNDS

As tricyclics are excluded, we considered those compounds which possessed an obvious structural resemblance to those antidepressants whose structures were characterized by a combination of a set of 3 fused rings and a basic chain attached to the middle ring (6). Tetracyclics which possess a clear structural rela-



(6)

tionship with the tricyclic compounds have also been excluded, but tricyclics which have scarcely any relation to the above type have been included. Also

excluded are groups of compounds which can be considered as belonging to special pharmacological classes by virtue of their mechanism of action. Concerning these groups the reader is referred to some recent reviews, particularly for the established monoamine oxidase inhibitors [34–37], for the thyrotrophin-releasing factor (TRH) and related peptides [38], and for compounds of lithium and other metals [39]. Although some antidepressant steroids with or without any endocrinological effects have been described (e.g. [40,41]), these compounds also have not been mentioned further.

A large number of non-tricyclics for which (pharmacological) antidepressant activity is claimed have been described in the last 20 yr, but only those structures are selected whose pharmacology has a pronounced antidepressant profile and/or in which effectiveness in human depression may be expected now or in near future. In general, compounds of which no clinical investigation has been reported more than about 5 yr after their announcement are excluded. In cases of doubt, we made inquiries of the investigators concerned about possibly predicted clinical usefulness. For the presentation of series, the most active or the most pronounced compound of the series is described.

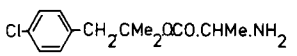
Although the patent literature often has a somewhat coloured character, this source could not be neglected. Many compounds and their activities have been described in patents some years prior to the publication of other scientific papers, while others are described exclusively in patients.

The structural classification used is based on functional groups or characteristic nuclei and is an arbitrarily chemical one. As most of the compounds are polyfunctional, that function is chosen which seemed the most characteristic. Analogous structures or compounds derived from each other have as far as possible been collected into groups. All structures are given in the non-salt form.

NON-TRICYCLIC ANTIDEPRESSANT STRUCTURES

CARBOXYLIC ACIDS, ESTERS, AMIDES AND AMIDOXIMES

An alanine ester, alaproclate (7) and some of its analogues selectively inhibit 5-



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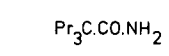


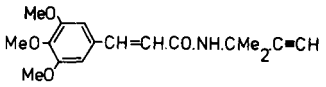
(8)

HT uptake *in vitro* and *in vivo*, and (7) potentiates 5-HTP. Cardiac arrhythmia is not observed, and peripheral anticholinergic activity is equal to or less than that of imipramine. The meta-chloro analogue is *in vivo* an even more active 5-HT

uptake inhibitor than (7) [42]. From the tentative pharmacophore and conformations of 5-HT and (7), a hypothetic carrier site for 5-HT uptake has been deduced in terms of geometry and electronic properties [43]. Compound (7) is undergoing open clinical trials in patients with mainly endogenous depression [44].

A simple aliphatic amide (8) and some alkali salts of the corresponding acid have been mentioned as possessing activity in depression and schizophrenia in humans [45].

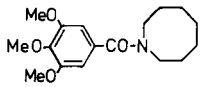
Compound A-25794 (9) was announced as an antidepressant (animal tests) at



(9)

low doses, being tranquillizing at higher doses, and possessing low toxicity. The compound had no MAO inhibiting properties [46]. However, therapeutically (9) seems to be not very effective [47,48].

Reserpine antagonism in rats and potentiation of reserpine-induced hypermotility due to the initial monoamine releasing effect in mice was found for N 1157 (10) [49–51].



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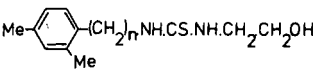
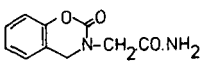


(11) n = 0
(12) n = 1

Some *N*-(2-hydroxyethyl)thioureas, e.g. (11) and (12), antagonized reserpine ptosis (rat) [52,53] and (11) prolonged hexobarbital induced sleeping time (mice) [53].

A kind of cyclic carbamate amide (which may also be classified as a benzoxazine or as a glycinamide) is caroxazone (13). This is pharmacologically

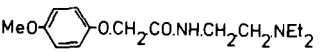


(13)

characterized by a strong anti-reserpine activity and by potentiation of the effects of NA and 5-HT [54–56]. Such effects are related not to an inhibition of the neuronal uptake of the monoamines [57], but to a MAO-inhibitory activity [58]. However, (13) differed sharply from the MAO inhibitors hitherto used in

therapy, as it shows an easy reversibility of its effect. This fact was invoked to explain why (13) in rats was found to be inactive on brain and liver mitochondrial MAO [56] but induced some modifications in 5-HT, NA, and DA concentrations in rat brain [58–60]. It differed from the tricyclic antidepressants also by lacking the anticholinergic and antihistamine effects [55,61], as well as by the absence of interference with the drug-metabolizing enzymes, aminopyrine demethylase and aniline hydroxylase [62]. Studies in healthy volunteers with therapeutic dose levels have confirmed the presence of a reversible MAO-inhibitory activity [63] and the lack of interference with drug metabolism [62]. In uncontrolled trials on 110 patients, 3 double-blind trials versus imipramine covering 30 inpatients and 4 double-blind trials versus amitriptyline (140 mainly in-patients), most of the patients suffering from a moderate to severe neurotic depression, (13) appeared as an effective drug. In one double-blind trial versus amitriptyline (5), carried out in 40 outpatients, (13) proved significantly better (total score) than (5) 3 days after the beginning, indicating a more rapid onset of action. In particular, (13) was significantly better than (5) with regard to depressive mood, work and interests, and suicide, while it was worse than (5) in the case of anxiety and agitation. The positive effect of (13) on these symptoms was even better than that of (5) at the end of treatment also, when the total score did not show any significant difference. Side-effects of mild to moderate degree and of short duration were seen in about 30–40% of the patients. No changes have been noted in laboratory tests, ECG, and blood-pressure [64–67].

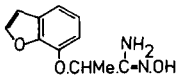
The basic amide, mefexamide (14), a representative of plant growth-regulating acids and derivatives, is included in this heading because of its amide function. The pharmacological effects of (14) corresponded with those of imipramine,



(14)

while (14) was relatively free from side-effects [68,69]. In uncontrolled clinical studies extending over 4 yr and involving 396 patients, (14) showed good results against depression in 55% of chronic alcoholics and in 80% of cases of tuberculosis, in severe neurotic depression (75%) and in melancholic states (50%). Very good results were found in 78% of the cases of reactive depression studied. The compound did not affect the cardiovascular system, nor did it possess other important side-effects. It is stated that (14) slightly depresses the gamma system (myotatic reflex and monosynaptic reflex) in small doses and activates it moderately in large doses, in contrast to the tricyclics and neuroleptics [70]. Apart from the antidepressive properties, facilitation of intellectual functions and psychomotor recuperation ('psychotonic action') were also noted [71].

A series of amidoximes, especially of benzofuran showed antidepressant properties in animals. L 7526 (15) possessed the highest activity in the reserpine



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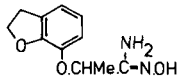
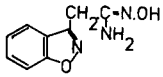


(16)

ptosis and hypothermia test (4 times that of imipramine) [72,73]. In general, the 3-position in these benzofurans was the most unfavourable location for the side chain. The compounds also had anticholinergic effects but were not MAO inhibitors [72]. In a probably open clinical trial with (15) involving 45 depressed patients, only 12 responded moderately, whereas various side-effects were noted. The dihydro derivative (16) had lower anticholinergic effects, but work with this compound had to be abandoned after adverse results in chronic toxicity studies [74]. Some, especially the α-naphthyl analogue, showed anti-aggressive effects in isolated mice [75].

Another amidoxime, PF 257 (17) antagonized reserpine in NA-depletion. It



(17)

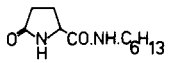
delayed NA turnover in the CNS without showing inhibition of MAO or of catechol-*O*-methyltransferase (COMT) [76–79], which may be the mechanism underlying the pharmacological effect [78]. One of the likely sites of action is stated to be in the caudate nucleus and the depression of this nucleus probably results in an increased susceptibility of the thalamocortical region [79].

The compounds of this category illustrate that neutral and acid compounds can also exert antidepressive effects and/or influence relevant biogenic amines. It is a rather small group in comparison with the basic compounds, but it may be concluded that basic groups are not always essential for antidepressant activity.

AMINO ACIDS

Although TRH, triiodothyroxine and analogues are excluded, some simple amino acids have to be mentioned.

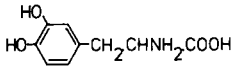
Some pyroglutamylamides showed activity in animal tests, e.g. (18) at 0.5 mg kg^{-1} in mice potentiated DOPA-excitation by 217%. The effect was comparable



(18)

with that of TRH [80]. Clinical investigation has been abandoned, since the antidepressant activity of TRH was not confirmed [81].

Of the 2 precursors of NA and of 5-HT, respectively L-DOPA (19) [82] and L-tryptophan (20) [83,84], applied in high doses, that of (20) seems to be the



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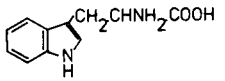
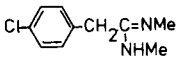


(20)

more successful as an antidepressant. In a review, L-(20) is stated to be useful in the treatment of the depressive phase of manic patients [85].

AMIDINES

A group of amidines (21–24) presented a pharmacological profile characteristic



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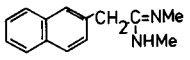

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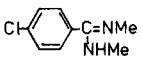

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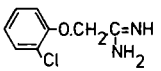
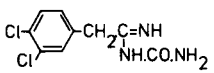


(24)

of antidepressants, especially in regard to reserpine antagonism [86–91]. In these tests, compounds (21) and (22) were active at 2–10 mg kg^{-1} (i.p., mice) and also counteracted electroshock-induced aggression, but at a much higher dose [86–88]. This group of amidines may be characterized as consisting of fairly strongly basic compounds in which lipophilicity is increased by substituents in the phenyl ring and at the basic function (21–23).

Also *N*-carbamoyl derivatives, e.g. (25), antagonized tetrabenazine at 2–

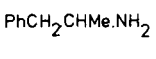


(25)

10 mg (i.p., rat) and showed low toxicity [92]. Because of toxic effects found later in some animal species, the substances were not developed to the clinical trial stage [93].

ARYLALKYL- AND ARYLCYCLOALKYLAMINES

A considerable proportion of this class of compounds is derived from the structure of amphetamine (26). By modifying this structure attempts were made



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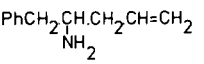

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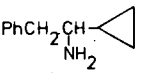


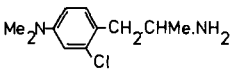
(28)

to remove the well-known motor stimulation effect of (26) and to obtain an antidepressant action.

One example of these structural analogues is aletamine (27), originally evaluated clinically as an analgesic [94]. It proved to be more active than imipramine in antagonizing reserpine. It lacked the antitremorine activity characteristic of the tricyclics, whereas the effects on spontaneous motor activity, hexobarbitone induced sleeping time, and NA-pressor activity were the opposite of those of (+)-amphetamine. Some evidence of mood elevation was found in healthy volunteers [95].

Another compound (28) of this 'amphetamine-type' antagonized reserpine in animals, did not cause motor stimulation nor inhibit MAO but still produced anorexia [96].

Related types are (29) and (30). The introduction of a para-dialkylamine



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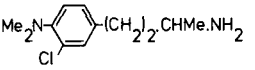
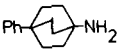


(30)

group resulted in compounds devoid of central stimulant action and even showing slightly sedative properties. They exhibited pronounced 5-HTP potentiation activity (ED_{50} 0.1–0.25 mg kg^{-1} i.p. mice) and antiagressive properties (ED_{50} 2.5–5 mg kg^{-1} i.p. in the isolated mice test) [97,98]. Of the (29) series, several were selective MAO inhibitors [99]. When the aromatic amino group and the phenyl ring form a tetrahydroquinoline system, activity is maintained [100].

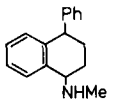
EXP-561 (31) may also be placed in this group. It had an antidepressant profile analogous to imipramine and in some respects (e.g. tetrabenazine

antagonism) (31) was much more potent than imipramine [101,102]. It was a



(31)

potent relatively nonselective NA-uptake blocker. Administration of (31) led to a reduction in uptake of both NA and 5-HT by the heart and by synaptosomes of different areas of the brain (rat) [103]. In open clinical trials, however, the value of (31) was doubtful: in depressed schizophrenics, a worsening of their condition was observed [101]. Another study mentioned only overt stimulation in ten cases of endogenous depression [102]. The imipramine-like action of (31) supported a hypothesis that the aminoalkyl side chain was folded towards the aromatic ring in the active conformation of the tricyclic antidepressants [104]. Further support of this hypothesis was provided by the high potency of the 1*R*, 4*S*-isomer of (32), CP-24,441 [104,105]. Based on compounds such as (31–33)



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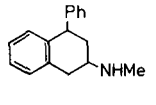
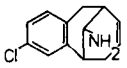


(33)

and also (129, 130, see continuation) with relatively rigid molecular structure, hypotheses are developed for the most appropriate conformation for reuptake inhibitory properties of NA and DA as well as 5-HT [106].

Another bridged compound which can be placed under this heading is ORG 6582 (34). In blocking the 5-HT-depleting action of *p*-chloroamphetamine in rat



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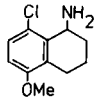


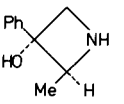
(35)

brain (*in vivo*), (34) was 5 times more potent than chlorimipramine and had also a longer duration of action. Whilst having no effect on amine steady state levels, (34) decreased rat brain 5-HT turnover and lowered 5-hydroxyindoleacetic acid (5-HIAA) levels, probably due to reuptake blockade. No effect on NA and DA metabolism was found [107,108].

Showing some structural relationship to (32–34) is (35), of which the *S*-stereoisomer showed antidepressant effects in animal studies, whereas the *R*-isomer was mainly α-lytic and lacked the antidepressant activity [109].

The preceding examples, especially (27–30), show that by small variations in the basic carbon chain (e.g. introduction of an unsaturated bond or ring closures) or by special substituents in the aromatic ring the motor stimulation of amphetamine (26) can be completely abolished.

Studies on conformation-restricted phenethylamines, showed that the NA-uptake inhibition activity of the trans isomer (36) is seven times that of the cis



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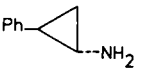
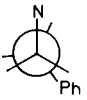


(37)

compound. This and the fact that the trans isomer (37) (tranylcypromine) is 600 times more active as the cis isomer in NA-uptake inhibition lead to the conclusion that an anticlinal conformation (38) of the $PhCH_2 \cdot CH_2 \cdot NH_2$ moiety is



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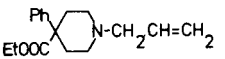


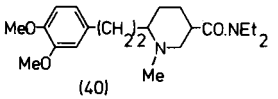
(39)

preferred in NA-uptake inhibition [110].

Some meperidine analogues, e.g. (39), were active in the DOPA-potentiation test while lacking the morphine-like properties of meperidine [111]. Clinical activity has not yet been reported.

Somewhat related, but with quite different functional groups is the basic amide (40) and its morpholine analogue. Both were as active as imipramine in the DOPA-potentiation test [112,113].

A promising compound of quite different type is 933CB (41). It is claimed



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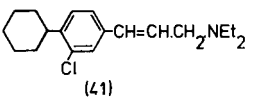

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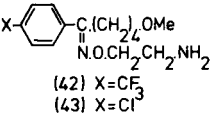


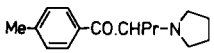
(42) X=CF_3
(43) X=Cl

that at 40–60 mg per day (41) gave positive results in 21 of 30 deeply depressed patients; of the 21 patients, 13 responded within 1 wk [114]. Some analogues of (41) are also under clinical trial, but nothing has yet been published [115]. In pharmacological tests, (41) showed an increase of motor and explorative activity and it antagonized reserpine-induced ptosis. Moreover it displayed an anti-cataleptic effect (catalepsy induced by prochlorperazine) and antagonized

reserpine ulcers (rat). In rabbits it augmented the post-discharges after hippocampal stimulation in the EEG by 60%. The oral toxicity was favourable [114].

Other quite different and promising compounds are represented by fluvoxamine (42) and clovoxamine (43). These compounds were developed from a large series of basic oxime ethers, some showing MAO inhibition [116], some sedative and anticonvulsive activity, and others pronounced antidepressant activity, without any MAO inhibiting effect. Anticholinergic effects and effects on the ECG were absent. Animal toxicological studies were favourable. Even small variations of the (2-aminoethyl) moiety reduced the tetrabenazine-antagonizing activity considerably [117–119]. In the antidepressive compounds, apart from inhibition of the uptake of both 5-HT and NA, also selective 5-HT uptake inhibitors were obtained, mainly by variation of the phenyl substituent [120–125]. Compound (42) is a selective 5-HT uptake inhibitor [15] which is under clinical trial. Compound (43), which is also being investigated in human depression, is an inhibitor of the reuptake of 5-HT as well as of NA. Effects on EEG in healthy persons show a typical antidepressant profile for (42) and (43), which resembles that of imipramine (1), but (42) has a longer lasting activity [126]. Results of double blind clinical trials in depressed patients are encouraging [127].

The introduction of keto groups in phenethylamines, in general also leading to potent psychostimulants and/or anorexants has provided some interesting compounds: in a study of 40 male law-breakers, the psychostimulant pyrovalerone (44) had a better effect on depression than did psychotherapy (double-



(44)

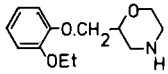
(45)

blind cross over; self rating depression scale). There were no signs of dependance and side-effects were fewer than with placebo [128].

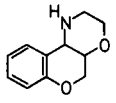
Bupropion (45) in early therapeutic studies was found to be active in chronically depressed patients, without giving rise to significant findings in standard laboratory tests and to ECG or EEG abnormalities. It was reported to have a rapid onset of action and to lack anticholinergic side-effects [129]. In a double-blind trial versus placebo in patients with endogenous depression, (45) was found to be active in the first week of therapy (Hamilton scale) with minimum side-effects [130]. The compound was not anticholinergic nor a MAO inhibitor, and, with the exception of pupillary dilatation, peripheral adrenergic activity was not apparent. In its capacity to antagonize tetrabenazine-induced sedation and ptosis, (45) was similar to amitriptyline (5). It differed from (5) in producing an increase in motor activity at doses 2 to 3 times higher than those required for tetrabenazine antagonism [129,131].

PHENOXYALKYLAMINES

A group which can be considered as cyclic analogues of the betalytics is formed by the basic phenoxyalkyl derivatives, represented by viloxazine (46). In (46), a



(46)



(47)

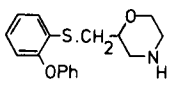
morpholine ring is formed with the alkylene chain, the amino group, and a hydroxy group already present in betalytics. Thus this type can also structurally be classified as morpholine analogues, but the analogues of (46) are summarized as phenoxyalkylamines. However, the presence of the heterocycle will no doubt have its influence on the pharmacological profile; the introduction of this group often leads to psychotherapeutic activity. Moreover introduction of an *O*-bridge in the alkylene chain might decrease possible stimulant effects. Compound (46) and its analogues are devoid of betalytic properties and have a pronounced effect on the CNS [132].

In several antidepressant tests, (46) is very active; the oral ED_{50} in reserpine and tetrabenazine tests is 0.3–1 mg kg^{-1} [133,134]. Compound (46), and also its meta-MeO analogue, with *S*-configuration, is at least 10 times more potent than the *R*-(+)-isomer in these tests [135]. Effects on biogenic amine uptake mechanisms in mouse and rat heart were half as potent as, and of shorter duration than, those of imipramine (1). Uptake of NA in rat medulla or hypothalamus was not inhibited by (46), but alteration of the NA-metabolite pattern was similar to that associated with (1) [136]. Clinically a rapid onset of action of (46) in depressed patients was claimed [137], but in subsequent trials only a minority adhered to this view; in general no difference from imipramine was demonstrated in various double-blind trials [132]. It appeared to have less peripheral anticholinergic and possibly a lower intrinsic activity in blocking the monoamine uptake process than (1). In depressed patients with clear anxiety components, (46) showed also favourable results [138].

By making the structure of (46) more rigid, as in (47), antidepressant activity in rodents was lost: neither cis nor trans (47) showed any activity [139].

Reserpine antagonism is also found in phenylthio analogues with ortho alkoxy or phenoxy groups, especially (48) [140,141]. In this series and also in analogous anilinomethyl morpholines, the SAR parallel those of the (46) series. The active aniline compounds are at least as potent as (46), the (48)-series are somewhat less potent [141].

Activity was also shown by some *m*-oxazinethiones, e.g. tifemoxone (49)



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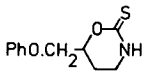
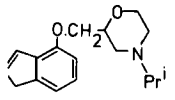


(49)

[142–144]. Compound (49) was found to be active in human depression with a moderate efficacy at high dose (600 mg per day), but in long term toxicity studies it exhibited antithyroid and hepatotoxic effects, after which studies stopped [145,146].

When the phenyl group of the (46)-series was replaced by an indenyl group, as in (50), the same type of pharmacological activity was obtained in combina-



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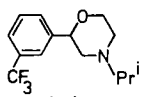


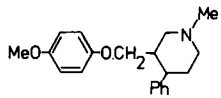
(51)

tion with low toxicity. Corresponding indanyl derivatives were inactive, indicating the importance of the unsaturated centre in the cyclopentene part of this type [147]. Neither (50) nor any of its analogues have been evaluated clinically [148].

Structural analogy with (50) concerning the morpholine moiety is presented by oxaflozane (51), but the ether bridge is lacking. In male patients this was shown to be an effective antidepressant in open trial, without cardiovascular side-effects [149,150]. In animals, it antagonized reserpine and did not inhibit brain 5-HT uptake nor MAO activity. It had a cataleptogenic effect and a very marked anti-aggressive action [151].

Since in (51) the morpholine ring is connected directly to the phenyl fragment, and as (51), in contrast to (47), is active, it may be hypothesized that the position of the morpholine ring in regard to the phenyl nucleus is essential in this type of antidepressants.

Additionally femoxetine, the (+)-trans form of (52), can be placed in the group of phenoxy derivatives. Animal studies indicated that it was a 5-HT up-



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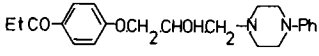


(53)

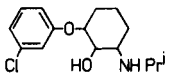
take inhibitor in the CNS, with relatively less influence on NA uptake. The

effect on the peripheral sympathetic mechanism was much less than that of the tricyclics. The peripheral anticholinergic effect corresponded with that of chlorimipramine, but the compound was devoid of peripheral antihistamine activity [152–154]. Toxicity was favourable in comparison with (1) but in a small double-blind study its clinical effectiveness was less than that of amitriptyline (5) [155].

Compounds of the betalytics type in which the hydroxy group is still present are (53) and (54).

In a series of analogues (53) showed a pronounced antidepressant profile. It counteracted reserpine (depression in mice and emesis in pigeons) and potentiated the action of amphetamine. The position of the alkanoyl residue seemed to be very specific. Small variations of the propionyl moiety abolished the antidepressant activity and (in some cases) gave other effects e.g. depressive with Et · CHOH- and hypotensive with $EtSO_2$- [156]. Compound (53) was well tolerated and showed no particularly adverse effect in humans in a 4-wk study at 40–80 mg daily. Clinical studies in patients have been started [157].

A series which strongly inhibits the 5-HT releasing effect of the compound H75/12 is represented by (54), which also has no cardiovascular effects [158].



(54)

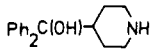
DIARYL AND DIARYLOXY DERIVATIVES

Structures showing an antidepressant profile and characterized by the presence of the 2 aryl or aryloxy groups, connected by a 1 (or 2) atom bridge, which also bears a basic chain, are now considered as a single group. Besides the basic function, a hydroxy group, an ether bridge, or an aliphatic ring may be built into the basic chain. The 2 aryl rings may be phenyl, but also (phenyl-, heteroaryl) and bis (heteroaryl) derivatives are included.

This structural system can be considered as related to the tricyclics, because in the latter also 2 aryl groups are present, but then as a rigid ring system. However, pharmacologically and also in respect of steric structure there are pronounced differences between this structural system and the tricyclics.

From the diarylalkyl type, different series with much variation in central effects can be derived. The sedative azacyclonol (55) and the psychostimulant piperadrol (56) illustrate that even small structural variations can change the pharmacological effects.

A large series of diphenylmethylene derivatives, which were developed from



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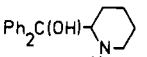
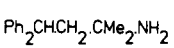


(56)

spiro-tricyclic compounds [159] and have been extensively studied are represented by (57–62). In these compounds the steric positions of the phenyl,



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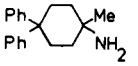
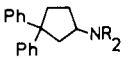
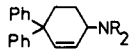


(58)



(59) R=H
(60) R=Me



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

alkylene, and amino groups is more or less restricted, while phenyl substituent effects are almost excluded.

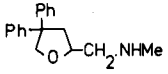
Compound (57) was the most active in inhibiting neuronal uptake of NA and 5-HT *in vitro* and (somewhat less pronounced) *in vivo* in a series with NH_2, NHMe, and NMe_2, and a variable chain length of 2–5 carbon atoms. It also potentiated the L-DOPA response (motor activity in mice) and induced a delayed onset of motor stimulation [160]. Also (58) and analogues showed considerable inhibition of NA-uptake in rat brain slices but were poor inhibitors *in vivo*. Compounds in this series have little or no effect *in vivo* or *in vitro* on 5-HT uptake [161].

Of 30 diphenylcycloalkylamines of types (59–62), several were active inhibitors of the neuronal uptake of NA and 5-HT (*in vivo* as well as *in vitro*) [162,163]. The primary amines (59) and (61) were the most potent in inhibition of 5-HT uptake, whereas the tertiary amines (60,62) were more active than the corresponding secondary amines. On the other hand the tertiary dimethylamino compounds (60,62) were the most active in NA uptake inhibition (*in vitro*) [162]. The enantiomers (–)-(59) and (+)-(61) were the most active with respect to their antipodes; (+)-(61) was also the least toxic. The structures of (59) and (61) are almost superimposable, which strengthens the indication that the mechanism involved is sensitive to stereochemical differences [164].

Compounds (57–62) and some analogues showed high anticholinergic activity whereas i.v. acute toxicity was lower than that of amitriptyline (5) [161]. Many of types (57,59–62), induced a delayed onset of motor stimulation. Apparently this is not the case with (58): it reduced motor activity at high doses [161].

No results of tests in humans of any of the compounds of this pharmacologically well-investigated group have been published up to now.

A series of the same type, in which 'bio-isostericity' with tricyclic antidepressants is suggested, is represented by (63). It antagonized reserpine in the



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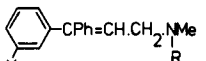
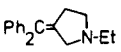


(64) X=F R=Me
(65) X=H R=H

same dose range as did demethylimipramine (DMI) [165]. The compound was not developed further for investigations in humans [166].

Under this heading also (64) should be mentioned, which was the most active in its series in inhibiting 5-HT uptake by human platelets (*in vitro*). It antagonized the effects of reserpine, but NA-uptake inhibiting *in vitro* data were not mentioned. The unsubstituted and the para fluoro isomer of (64) were also active [167]. With the -NHMe analogue BW 247 (65) a study of the effect on the EEG of healthy persons has been reported [168].

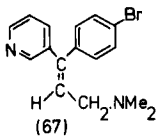
Closely related to this type is AHR 1118 (66), which based on reserpine



(66)

antagonism and EEG data (cats) combined with only weak anticholinergic effects, was developed as an antidepressant [169]. In a double blind study in patients with mainly severe depression with motor retardation, no overall difference was found between the therapeutic effects of (66) and of amitriptyline (5). Yet there were slight differences suggesting that the effect appeared earlier [170]. Other clinical studies with (66) have been planned and various analogues, some with higher activity, are being investigated [171].

Replacement of phenyl by 3-pyridyl in this type resulted in an interesting pair of compounds: (67) and (68). The Z-isomer, zimelidine (68) and its mono-



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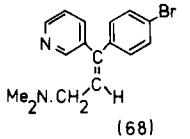
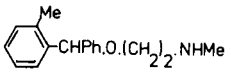


(68)

methyl analogue inhibited 5-HT uptake both *in vivo* and *in vitro* at much lower concentrations than that needed for inhibition of the uptake of NA. In contrast

to this, the selective inhibition was reversed in the E-isomer (67) and its NHMe analogue. These were very active inhibitors of NA uptake but they had only weak influence on 5-HT *in vivo* [172–177]. *In vivo*, (68) is rapidly dealkylated to the secondary amine (also in man) [175]. By means of biochemical methods, (68) was shown to be a selective 5-HT uptake inhibitor in depressed patients also. A good correlation existed between the 5-HT uptake inhibition and the plasma concentration of demethylated (68) but this correlation was not found with (68) itself [178].

Introduction of an ether bond in the alkylene chain is realized in tofenacin (69), which is an inhibitor of NA and 5-HT uptake by striatal synaptosomes



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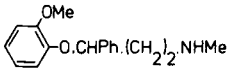


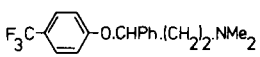
(70)

[179]. In an open label study in humans it was well tolerated [180,181]. In a double-blind trial it proved more successful in neurotic and mixed than in endogenous depression [182]. The dimethylamino analogue of (69) is the anti-Parkinson drug orphenadrine [183].

By introducing an ether bond between one phenyl ring and the alkylene chain in the diphenylmethylene derivatives, one obtained a series containing selective inhibitors of NA and of 5-HT as well as of DA [184]:

Nisoxetine (70) in man produced minimum side-effects and markedly diminished the pressor effect of tyramine and enhanced that of NA. *In vitro*, (70) inhibited 5-HT uptake in platelets, but platelets obtained from subjects receiving chronically administered (70) accumulated 5-HT. Plasma concentrations of (70) in these subjects was lower than that needed to block 5-HT uptake [185–188]. Thus in humans it seemed not to be a 5-HT uptake inhibitor. Specificity *in vivo* in NA reuptake inhibition was demonstrated by the antagonizing effect on 6-hydroxydopamine-induced depletion of NA in mouse heart [189]. It suppressed REM-sleep in cats and induced a sleep pattern resembling that produced by tricyclic antidepressants [190]. It is currently being evaluated in depressed patients [191].

In contrast to (70), fluoxetin (71) was a potent 5-HT uptake inhibitor, both



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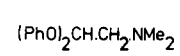


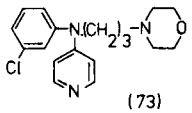
(72)

in vitro and *in vivo*, and did not block NA uptake. The NH_2 and NMe_2 com-

pounds were less active than the NHMe derivative as 5-HT uptake inhibitors [192–199]. The inhibiting effect on food consumption was short-lasting (2 h) and the anorexigenic effects of 5-HTP during that time were potentiated by (71) [200], another indication of 5-HT potentiating activity. In normal volunteers (71) does cause inhibition of 5-HT uptake by platelets at doses that do not lead to side-effects nor to perceptible changes in behaviour [201]. It has not yet been tested in patients [191].

A related diphenoxy compound, medifoxamine (72), was announced as being an imipramine-like antidepressant with no anticholinergic action [202]. It inhibited synaptosomal NA-uptake [203]. Attempts were made to correlate its dipole moment, UV spectra and pharmacological activities with possible conformations. Based on these data, there was support for the hypothesis that the various conformations of (72) corresponded to various biological activities, in particular a sympathomimetic, a spasmolytic, an antihistaminic, and an anti-serotoninergic activity [204].

Replacement of the carbon atom between the 2 aryl groups by nitrogen has been realized in (73). In a clinical uncontrolled study of 18 mth with

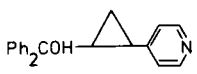


(73)

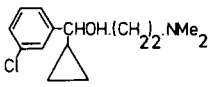
(74)

100 patients (73) proved to be anxiolytic with antidepressant properties in serious neurotic patients [205]. Hydroxy groups have been introduced at different sites in the alkylene chain, but especially at the linking carbon atom. An example is U 23807A (74). The presence of the methyl group in (74) minimized the anticholinergic activity in a series of analogues [206]. In an uncontrolled study the active (–)-enantiomer of (74) showed a doubtful antidepressant effect in neurotic depressions and caused visual hallucinations and dizziness [207].

Cyprolidol (75), another representative with an OH group, showed a



(75)

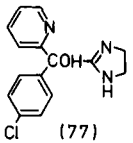


(76)

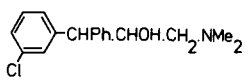
pharmacological profile corresponding with that of imipramine (1), except that in anaesthetized dogs it blocked the tyramine-induced pressor effect but potentiated it in conscious dogs [208–212]. In patients it was well tolerated and effective in a small trial [208], but in double-blind studies proved less effective than (1) in endogenous depression [213].

As cyclopropyl groups show some unsaturated character, compounds of type (76) are included in this class. Compound (76) and some analogues block tetrabenazine induced effects at low doses (2.5 mg kg^{-1} i.p. in the case of (76) [214,215]. However, none of these have yet been tested clinically [216].

SCH 12650 (77), a pyridyl imidazole derivative, is also placed in this class. It



(77)

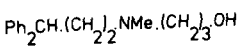


(78)

shows an antidepressant profile in animal tests [217,218], but is not being pursued clinically [219].

Diaryl derivatives with a hydroxyl group at another site in the alkylene chain are represented by BRL 14342 (78). Some structural relationship with (76) and (77) may be seen in this compound, which is currently undergoing tolerance and EEG studies in human volunteers. The results suggest that (78) is a potent non-depressant CNS-active drug [220]. The compound is a 60 : 40 mixture of the two possible diastereoisomers (racemates?), which are of approximately equal activity [220]. It is stated that the (−)-isomer shows fewer peripheral anticholinergic effects than the (+)-form. The (+)-isomer is more active than the (−)-isomer in the reserpine reversal test at low doses, but the 2 isomers have roughly equal effects in tests on prevention of reserpine-induced hypothermia [221]. Because (78) has 2 chiral centres it is not clear if the optically active compounds are single diastereoisomers or mixtures of 2 of them.

PF 82 (79) also contains OH, but on the *N*-alkyl substituent. It effectively



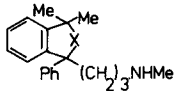
(79)

prevented reserpine induced hypothermia and ptosis and inhibited synaptosomal uptake of NA to the same degree as (1). The anticholinergic effect was less than that of imipramine (1) [222]. The preclinical evaluations in animals have been completed and these indicate that (79) is less toxic than (1) in subacute tests in rats and dogs [223]. In healthy volunteers no appreciably adverse effects were observed in a variety of tests, including subjective complaints and objective functional or biochemical findings [223].

For (76–79) no data of treated patients have appeared, but from the published data, (79) seems interesting.

ARYLBICYCLIC COMPOUNDS

From the preceding diaryl compounds, bicyclics of type (80–82) are obtained by connecting a part of the aliphatic chain with one of the aryl rings. The

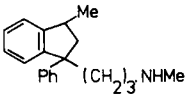


(80) X=O
(81) X=S
(82) X=CH_2

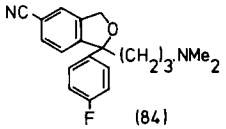
properties of this type of compound stimulated the investigation of several series. Talopram (80) and talsupram (81) were already announced in 1966 as possible antidepressants, based on their pharmacology, especially their action on the reuptake mechanism of the central and peripheral catecholamine neurons combined with absence of anticholinergic, MAO inhibiting and amphetamine like activities [224–231]. Kinetics in dogs were studied [232].

Lu 3-049 (82) possessed a potent and selective inhibitory effect on NA uptake comparable to DMI in rat brain and mouse heart. However, DMI induced a marked increase in brain NA-turnover and (82) caused little or no change [233].

Lu 3-057α (83), a monomethylindene, showed a corresponding profile [224–226]. The dimethylamino analogue also possesses pharmacological antidepressant activity.



(83)

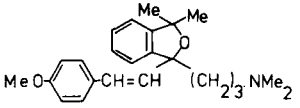


(84)

A number of the compounds inhibited mitochondrial activity as measured by yeast cell growth. Some were 3 or 4 times more potent than (1) in this test, in particular (82) and Lu 3-047, the dimethylamino analogue of (81) [234,235]. Notwithstanding the extensive pharmacological investigation, no clinical data have appeared yet.

Recently citalopram (84) was announced as a potent specific potentiator of 5-HT, being 5–10 times as active as chlorimipramine and active both *in vivo* and *in vitro*, probably due to inhibition of 5-HT uptake. It does not possess the NA potentiating, anticholinergic, and antihistaminic properties characteristic of the tricyclics [236–238]. The compound is in an early stage of phase I clinical studies [239].

It is not surprising, that after the first publications of these interesting series, related structures should have been synthesized by other workers. In these analogues a series of cinnamyl derivatives showed high activity: compound (85) was as potent as (80) in tetrabenazine antagonism [240].



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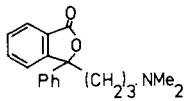
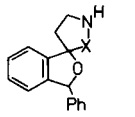


(86)

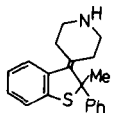
Another example is I.C.I. 53,165 (86), which increased turnover of brain NA and not of DA or of 5-HT (rats) [241].

Still another related group is represented by (87) and (88) which showed high



(87) X=CH_2

(88) X=$(CH_2)_2$



(89)

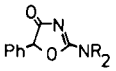
activity in antagonizing tetrabenazine ptosis [242,243]. They were active inhibitors of NA, 5-HT and also DA re-uptake (*in vitro*) [244]. A SAR study showed that large N-substituents reduced activity. Optimum (anti-tetrabenazine) activity was associated with the presence of the phenyl group: on removal of this group, activity was lost, and substitution of the remaining H-atom of the furan ring greatly reduced activity [243]. Corresponding N-OH derivatives of (88) also showed high activity in the tetrabenazine ptosis test [245] as did the *N*-propargyl derivative [246]. HP 505 (88), whose ED_{50} (i.p.) in the tetrabenazine test was 0.5 mg kg^{-1} was selected for further studies [243]. Compound (88) and its para fluoro analogue proved to be active in depressive patients [244].

Finally HT 3261 (89) can be mentioned; this was announced as being effective in humans in a probably open trial [247], but it was not further investigated [248].

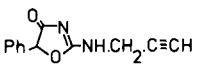
OXAZOLES, IMIDAZOLES AND TRIAZOLES

Although some of these structures show a relationship with one of the above mentioned groups, it seems appropriate to present them as a separate class.

The stimulants thozalinone (90) and pemoline (91) might have been proto-



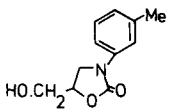
(90) R=Me
(91) R=H



(92)

types of this class. However, (90) proved to be no better than placebo in patients with reactive or endogenous depression [249]. The propargyl derivative (92) was the most active in a series in the DOPA-potentiation test with an effect lasting longer than 24 h. No effect on the monoamine levels in the CNS was found, indicating that it was not a MAO inhibitor in spite of the structural relationship to the MAO inhibitor pargyline (93) [250].

(93)

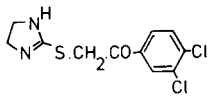


(94)

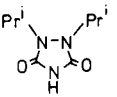
Toloxatone (94) antagonized reserpine and potentiated the effects of tryptamine and 5-HTP but not of DOPA. It had no amphetamine like, anticholinergic, antihistaminic or MAO inhibiting properties [251–253]. A later study showed that the compound was characterized by two agonistic effects: activation of tryptophan hydroxylase (*in vitro*) and an inhibition of MAO activity in the rat brain stem, with 5-HT used as a substrate. A possible interaction in the catecholamine pathways was not excluded [254].

Some other oxazolones have been described, but clinical data have not been published for these. From the series mentioned in a recent paper [255], not one has undergone clinical trial [256].

Of the imidazoles, the imidazoline (95) should be mentioned. It is one of the



(95)



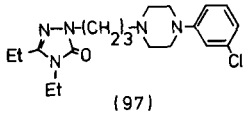
(96)

most potent reserpine antagonists in a series of 80 derivatives which were only weakly anticholinergic and which were studied further. Unfortunately, in contrast to studies in rats, it was embryotoxic in rabbits [257].

Clinical effectiveness in depressive patients (double-blind versus imipramine)

was found for the urazole, A 10749 (96) [258–261]. Pharmacologically (96) was effective in the DOPA potentiation test and was not a MAO inhibitor [258].

Another antidepressant triazinone is ST 1191 (97), an analogue of trazodone (98). In depressive neurosis, (97) caused more rapid and marked improvement



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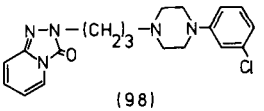


(98)

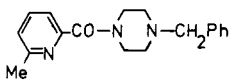
than imipramine. Moreover it alleviated symptoms of anxiety, hypochondria and obsession. It reduced anorexia and was well tolerated (40 patients, double-blind versus (1)) [262].

The older trazodone (98), composed of a related heterobicyclic and the same piperazine side chain as in (97), showed a complex pharmacological profile. Tranquillizing as well as antihypertensive properties were found in animal experiments [263]. A selectivity towards uptake inhibition of 5-HT was noted [264]. In controlled studies it proved as effective as DMI in depressive symptoms, but it had a rapid anxiolytic effect, which was not obtained with DMI [265].

PIPERAZINES

The previously mentioned (97) and (98) might also be classified in this group. Piperazine derivatives (as with the morpholine analogues) take a prominent place in CNS-active drugs.

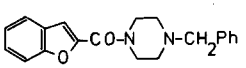
In a series of 27 *N*-(pyridylacyl)piperidines, (99) was the most active in



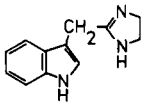
(99)

antagonizing tetrabenazine and reserpine effects, and toxicity was favourable [266].

Closely related is DIV 154 (100), which in the first, probably open, clinical



(100)



(101)

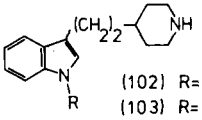
investigation was well tolerated and illustrated an interesting alternative in the treatment of various depressive conditions [267]. Pharmacologically, it was a potent reserpine, tetrabenazine and perphenazine antagonist at doses which did not affect the normal gross behaviour of animals. Without being sedative, it reduced fighting behaviour (mice). It also had anticonvulsive properties, was not anticholinergic and showed phosphodiesterase inhibiting activity [267].

INDOLES, BENZPYRAZOLES AND BENZIMIDAZOLES

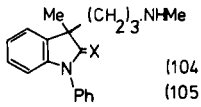
As indole derivatives, especially 5-HT, exert a distinct effect in the CNS, it is not surprising that many trials in the search for new CNS drugs should have been based on these structures.

A very potent reserpine antagonist (ED_{50} = 0.23 mg kg^{-1} p.o.; LD_{50} = 52 mg kg^{-1} p.o.) is found in (101) [268]. A potentiation of amphetamine-induced toxicity in aggregated mice was also described for (101), but further data are lacking.

A strong inhibition of 5-HT accumulation in rat brain synaptosomes and



(102) R=Me
(103) R=H

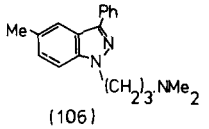


(104) X=O
(105) X=H_2

human blood platelets was reported for LM 5005 (102) and LM 5008 (103). No effects on 5-HT release from human blood platelets and no inhibition of *in vitro* uptake of NA into rat heart were present [269]. 5-HT turnover, as measured by rat brain 5-HIAA levels, was decreased. Brain tryptophan levels were raised by (102) and slightly lowered by (103) [270].

N-Phenyldihydroindoles, in which there is also a structural relation to type (80–88), are the antidepressants amedalin (104) and daledalin (105). Clinical effectiveness was ascribed to both [271–273], but (104) gave scrotal and urination discomfort [272]. Compound (105) was as effective in depression as amitriptyline (5) but caused headache in 6 of 38 patients studied [273]. The pharmacological profile of (104) showed potent antagonism of reserpine induced hypothermia and tetrabenazine-induced sedation in rats. In dogs, a potentiation of NA pressor response was obtained by 1 mg kg^{-1} p.o., which can be attributed to a neuronal uptake inhibiting effect of (104) [271,274]. In their series, (104) and (105) were the most potent in potentiation of adrenergic mechanisms [274].

Structural relationships to compounds of type (80–88) and to the *N*-phenyldihydroindoles are shown by FS 32 (106). It antagonized reserpine hypothermia



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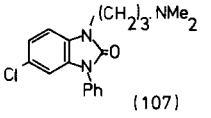
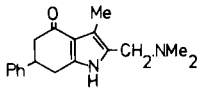


(107)

and showed favourable (oral) toxicity (mice and rat) [275]. Clinical trials are in progress [276].

Related to (106) is the older clodazone (107). The first clinical trials confirmed the pharmacological findings: a positive antidepressant effect in two thirds of the patients and remarkably few side effects [277]. Further trials confirmed the efficacy [278,279]. In an open trial in patients with retarded depression, some psychomotor activating effect was noted [278].

EN 3022 (108) was the only member of its chemical series that antagonized



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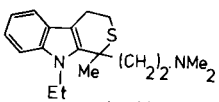


(109)

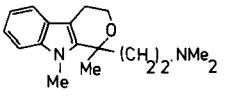
tetrabenazine ptosis and hypothermia (mice) but potentiation of DOPA was not seen in mice or rats. It potentiated NA and inhibited tyramine effects (anaesthetized dogs) and was not a MAO inhibitor (*in vitro*) [280,281]. It has not been investigated in humans [282].

CONDENSED INDOLE DERIVATIVES

Different tri- and tetracyclic compounds with an indole moiety in their ring system and possessing interesting effects on the CNS have been described. Among these are also several types of antidepressants.

Extensive studies have been published of compounds of the series of tandamine (109). This is a specific NA uptake inhibitor, more active than DMI. The (−)-enantiomer was the active component in the racemate; unfortunately the absolute configuration is not mentioned. The tertiary dimethylamino compound (109) is more potent than the secondary amine whilst the primary amine is inactive [283–286]. Whereas only weak anticholinergic activity in animals is reported [283], a small double-blind trial in healthy volunteers showed significant anticholinergic effects [287]. Clinical effectiveness in depressed patients has been demonstrated recently [288].

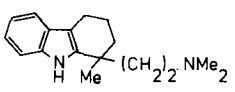
The related (110), a representative of a series of 24 oxygen analogues also showed inhibition of NA uptake but at lower potency than (109). A pronounced



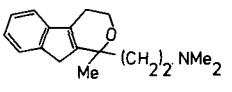
(110)

potentiation of 5-HT was found with (110), indicating that it also inhibited 5-HT reuptake [284,289]. Both (109) and (110) are very active antagonists of reserpine [284,286,289]. Between the enantiomers of (110) there was no difference in potency in these tests [289].

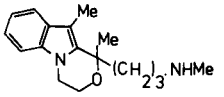
Also AY 24614 (111), the most active in its series, was considerably more



(111)



(112)



(113)

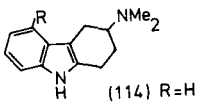
potent than (1) in antagonizing reserpine and tetrabenazine effects [290].

Although it is an indene instead of an indole derivative, the structure of pirandamine (112) is so closely related to (109–111) that it is mentioned here. In contrast to (109), this indene derivative (112) is a selective 5-HT uptake inhibitor. It did not show central anticholinergic effects in animals [283,285,291,292].

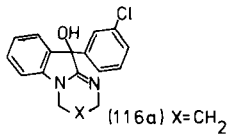
Comparison of the effects of these compounds shows again that in closely related series either selective NA or 5-HT uptake inhibitors are found.

Of a series of 'angular' homologues, AY 23673 (113) was the most active in antagonizing reserpine effects (12 times more than (1)). Only small variations in structure are allowed in maintaining the effectiveness. The activity is decreased on lengthening of the side chain as well as on increasing the bulk of the N-substituent. Replacement of the methyl groups in the tricyclic ring system by ethyl or propyl groups also diminishes the activity [293–295].

A simple carbazole derivative, WIN 27, 147-2 (114) showed clinical efficacy without side effects in a small uncontrolled study [296]. Compound (114)



(114) R=H
(115) R=Me

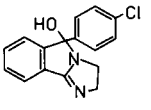


(116a) X=CH_2
(116b) X=$(CH_2)_2$

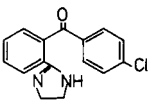
prevented reserpine induced ptosis and also amphetamine induced stereotyped behaviour in rats, indicative of neuroleptic activity. This neuroleptic factor was

not shown by (115), which was equally active in the ptosis test [297].

WY-23409 (116a) was as active as (1) in inhibiting NA uptake by rat cerebral



(117)

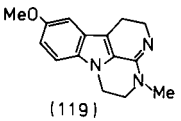


(118)

cortex slices, but 5-HT uptake was inhibited very weakly. In a variety of pharmacological tests, (116a) was only active in those indicating antidepressant activity, but there were differences between the profiles of (116a) and of (1)-like drugs (lack of anticholinergic effects, MAO inhibition and cardiotoxicity) [298,299]. Also the diazepine homologue (116b) was at least as active as (1) in NA uptake inhibition [300].

There is a strong structural resemblance of (116a) to the anorectic compound mazindol (117). This resemblance is also found in the NA-uptake inhibiting properties: (117) is a potent blocker of NA-accumulation of the rat heart *in vivo* [301]. Compound (118), obtained by protonation of (117), is an even more potent NA uptake inhibitor, and also shows strong DA and 5-HT uptake blocking properties *in vitro* [106].

Of incazan (119), clinical effectiveness in depression accompanied by a



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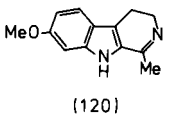
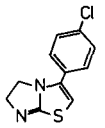


(120)

stimulant effect was reported [302]. In pharmacological tests, (119) potentiated many adrenaline like compounds and antagonized reserpine, tetrabenazine and phenothiazine. It has no anticholinergic, depressive, analgesic or local anaesthetic properties [303]. Notwithstanding structural analogy with harmaline (120), data on MAO inhibition were not presented.

CONDENSED THIAZOLES

The bicyclic compound (121) may be regarded as a more rigid derivative of type (95) and may also be looked upon as a cyclic isothiourea derivative. Like (95), compound (121) antagonized also reserpine and potentiated DOPA (mice) [257]. Related to (121) is a series of acetic acids and esters represented by (122),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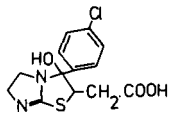
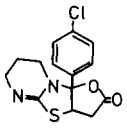

(122)

which is also a potent reserpine antagonist (ED_{50} = 0.2 mg kg^{-1} p.o.) [304].

A corresponding lactone (123), showed similarly high activity (ED_{50} =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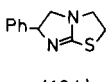
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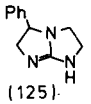
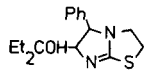

(125)

0.54 mg kg^{-1} p.o.). At a much higher dose (100–200 times), (123) reduced motor activity and respiration; it induced ataxia and loss of the righting reflex [305].

With the anthelmintic tetramisole (124) some mood elevating activity in depressed schizophrenics was observed during a double-blind crossover study [306,307]. The (+)-enantiomer of (124), dexamisole, showed effects in depression in small preliminary clinical trials [308]. The (–)-antipode, levamisole, was the active anthelmintic in the racemate [309–311], but of its central activity nothing is mentioned. The *R*-configuration was established for the (+) form [310]. Pharmacologically (124) had only a few of the attributes of the existing antidepressants [306,307].

Replacement of S in (124) by N resulted in a related group of bicyclic imidazoles and pyrimidines represented by imafen (125). It showed high activity in antidepressive animal tests (effective dose range 0.1–0.3 mg kg^{-1} s.c.). The (+)-enantiomer of (125) was 16 times more active than its antipode (tetrabenazine antagonism) [10,312]. The imidazothiazole (126) also showed high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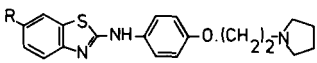

(127) R=H
(128) R=OMe

antidepressive activity in animals [313].

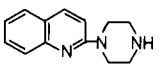
The benzthiazoles (127) and (128) were found more active than (1) in the reserpine reversal tests, but they also caused a state of alertness and increased sensitivity and some weak anticholinergic effects [314,315]. In contrast to the

benzoxazole analogues, (127) and (128) were not teratogenic or amphetamine-like stimulants [315].

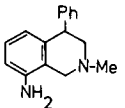
Clinical results have not been published for these compounds except for (104), (105) and the established drugs (107) and (124). Data were not available for compound (127) at the research centre concerned [316].

QUINOLINES AND TETRAHYDROISOQUINOLINES

The pharmacological profile of a simple piperazine derivative of quinoline,



(129)

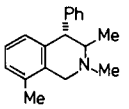


(130)

quipazine (129), corresponded with that of the tricyclic antidepressants [317]. However, during early trials in humans a strong emetic effect was discovered [318] and no further clinical trials were reported.

Nomifensine (130) is a tetrahydroisoquinoline possessing an antidepressant profile in animal tests, without cardiovascular or anticholinergic effects [319–322]. It is a potent inhibitor of DA-reuptake, but to a less extent it blocks also the uptake of 5-HT and NA [321–325]. In healthy subjects, (130) gives an EEG typical of antidepressants [326]. In an open clinical trial with 10 patients with mainly unipolar depression, seven showed much improvement [327]. Moreover a promising antiparkinson effect was noted in Parkinson patients [328].

Another structure of this type is the isoquinoline (131), one of a series of



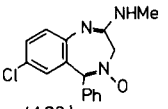
(131)

which the antireserpine activity was equivalent to that of (1) [329]. Activity of (dia)stereoisomers is not known.

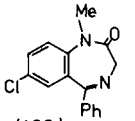
Structures of (130) and (131) are comparable with those of (31–33) for a hypothesis about appropriate conformations for optimum reuptake inhibition (see (31) etc.).

BENZAZEPINE-LIKE STRUCTURES

Although several compounds of this structural type find application in mental diseases, including some types of depression, most are of the tranquillizing type, with clordiazepoxide (132) and diazepam (133) as the most important representatives.

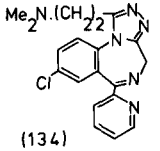


(132)



(133)

Of this benzodiazepine type, (134) has been announced merely for treatment of depression, it possesses minimum anxiolytic effect [330,331].



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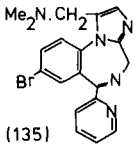
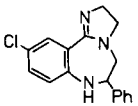


(135)

A shift to antidepressive properties in the benzodiazepines was also described for (135). Reversal of oxotremorine-induced hypothermia, inhibition of yohimbine aggregate toxicity and potentiation of apomorphine gnawing were used as antidepressive test. Anti-anxiety activity, as measured by prolongation of hypoxic survival time, and anticonvulsive properties were, however, also quite strong [332].

A group of analogues, e.g. (136) showed depressant activity in mice and



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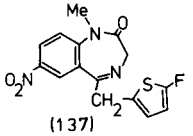
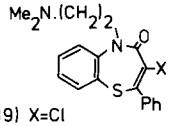


(137)

cats, but these compounds also antagonized reserpine ptosis [333]. Effectivity in deep depression in humans in an open trial was mentioned for LA-XIV (137) [334].

Of the benzthiazepines, thiazesim (138) was claimed to have a pronounced antidepressant effect in humans [335–337], but in a later study this activity was strongly criticized [338]. Its pharmacological profile does not indicate any anti-

(138)

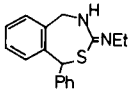


(139) X=Cl
(140) X=Br

depressant effect but in animal anti-aggressive models (septal and muricidal rats), however, (138) was active [24,339–342].

Of the chloro derivative (139) and its bromo analogue (140), a high activity in tetrabenazine antagonism was claimed [343], but up till now no clinical results have appeared.

A series represented by the thiazepine (141) antagonized tetrabenazine, induced ptosis and catalepsy and showed antiarrhythmic properties [344].



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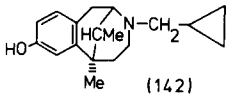
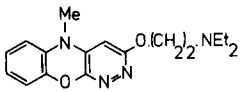


(142)

A bridged benzazocine, the analgesic cyclazocine (142), with scarcely any structural relation with this group, showed reserpine and tetrabenazine antagonism [345,346]. There was clinical improvement in patients with depression in an open trial, but a narrow therapeutic range has been mentioned [347].

REMAINING TRICYCLICS

A patent describes extensive clinical investigation concerning the benzoxazine (143): in 469 patients, most with mild to moderate depression in a probably



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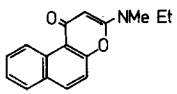


(144)

open trial, 60% of the patients showed clear improvement, 25% showed some or merely a temporary effect, and in 14% no effect was found. It had little or no effect against deep long-term depression. In animals, (143) reinforces central effects of amphetamine and reduced reserpine depression and phenothiazine induced catalepsy. It was neither anticholinergic nor MAO inhibiting. Oral toxicity was low [348].

Clinical effectiveness was also reported for K 8409 (144). In animals the compound antagonized reserpine and was not anticholinergic, it did not decrease REM-sleep or inhibit 5-HT uptake, and its cardiotoxicity was much weaker than that of the tricyclics [349]. In mice, anti-aggressive activity was noted. It clearly showed selective MAO inhibition; particularly in the brain, 5-HT oxidation was inhibited, but it had only little effect on the oxidation of tyramine in the liver [350]. In human volunteers it was well tolerated, but trials in patients with mild reactive depression demonstrated only little psychostimulation, not statistically different from placebo effect, whereupon the development was discontinued [350].

STRUCTURE-ACTIVITY RELATIONS (SAR)

For individual groups of compounds some SAR comments have already been given. These were mainly based on the investigators' own comments.

A survey of all the structures collected here makes it tempting to try to find some common physical or chemical characteristics which might be responsible for the biological action or even to establish the essential structural requirements for antidepressant activity.

This task, however, is seriously hampered by the following factors:

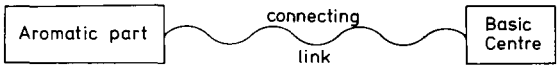
(a) the heterogenicity of animal tests and of clinical trials,
(b) a poor correlation between pharmacological data and clinical results
(c) mainly active structures have been presented,
(d) information about less active analogues in the series is often incomplete.

Inclusion of inactive compounds in this review would give more information about the limitations of structure, but they would overshadow the active ones and overcrowd the presented material. Nevertheless, with the above reservations in mind, some tentative suggestions of a general nature can be put forward.

MINIMUM REQUIREMENTS FOR ACTIVE STRUCTURES

The major part by far of these active structures consist of an aromatic, often lipophilic, moiety combined with a basic centre at a limited distance (145). Basic centres are mainly NH_2, NHMe, NMe_2, piperidyl and morpholyl. It seems that more bulky N-substituents diminish or prevent activity.

In some series, the tertiary amines are by far the most active, e.g. (84), (109), while in others the primary NH_2 derivatives are the highly active ones, e.g. (42), (59). These differences in activity are mostly based on results of pharmacological tests. The interpretation of *in vivo* experiments is hampered by the fact that *N*-



(145)

dealkylation of tertiary and secondary amines is an important metabolic factor *in vivo*. For instance, fluoxetine (71) is rapidly *N*-demethylated *in vivo* by rats and dogs to form the primary amine, itself an active metabolite [351].

However, on the basis of *in vitro* results it is often possible to deduce whether the metabolite(s) or the intact drug is the more active at the place of interaction. It might be that the physical-chemical properties, e.g. steric influences, of the connecting link play a role in defining these activity differences between differently substituted amines. A comparison of the activities of structures with a less or more fixed position of the basic centre, e.g. (46) and (47), makes it clear that this position has to fulfil certain steric requirements. With drug receptor interaction as the model, this is not surprising. It might also explain the lower activity of compounds with larger N-substituents.

UPTAKE INHIBITION AND STRUCTURE

When concentrating further on what is nowadays most in favour as the proposed mechanism of action, that of re-uptake inhibition, we come to more comparable biological parameters. The inhibition of transmitter uptake by different structures can be effectively measured with *in vitro* systems (see section on test methods) in which the effect is not influenced by possible pharmacokinetic parameters.

Based on *in vitro* results, quantitative differences within a series of compounds have been discussed in connection with the series concerned. Selectivity towards uptake inhibition in NA or 5-HT systems can also be seen in these series. The selectivities thus disclosed are not dependent on the test techniques followed, and selectivity differences can be compared in respect of various series. Some of these comparisons have already been made and proposals for optimal conformation suggested, e.g. [106]. The selective inhibitors discussed in this review (some of which are listed in pairs) are summarized in *Table 6.1.*

From this comparison some general conclusions can now be drawn:

(a) Often in the same series selective 5-HT as well as selective NA uptake inhibitors are found, which might be indicative of a related interaction with the relevant neuronal uptake systems.

(b) The selectivity of the inhibiting effect on NA re-uptake and that of 5-HT is mainly influenced in 2 ways: by variation of the substituent(s) in the aromatic part and by variation of the position of the basic centre, caused by fixation or substitution. Concerning the aromatic substituents it

Table 6.1. STRUCTURES WITH UPTAKE INHIBITION PREFERENCE [a]

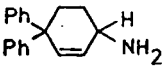
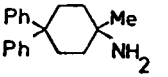
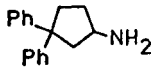
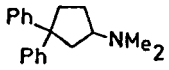
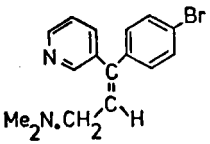
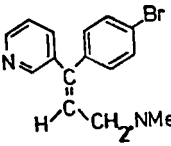
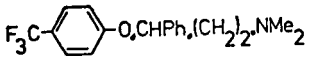
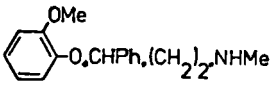
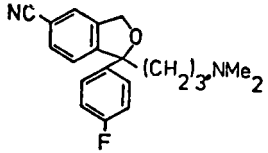
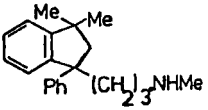
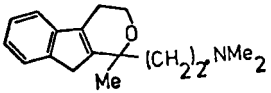
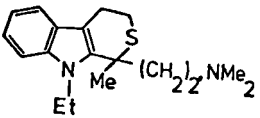
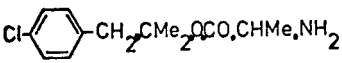
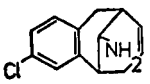
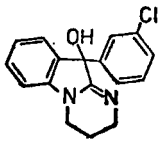
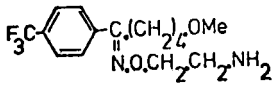
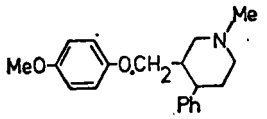
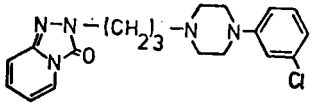
(No)	*for 5-HT*	*for NA*	*(No)*
61			58
59			60
68			67
71			70
84			82
112			109
7			
34			116a
42			

Table 6.1 (continued)

(No)	for 5-HT	for NA	(No)
52			
98			

[a] Based on *in vitro* and/or *in vivo* measurements

seems that 5-HT uptake inhibition is favoured by substituents with an electron withdrawing effect and limited steric size, especially CF_3, e.g. (42), (71). This substituent influence on NA is less clear. Concerning the basic centre it may well be that a more restricted position leads to a greater selectivity, e.g. (58), (60). In the selective NA uptake inhibitors this position seems still more restricted than in the case of 5-HT, e.g. (58) versus (59), (61) and (109) versus (112).

(c) In many cases structural analogy with either NA or 5-HT seems not to be of relevance, e.g. (58), (116a).

(d) By comparing the activities of individual stereoisomeric forms, the most favoured proposition is that a special steric arrangement is necessary for activity, e.g. (67) and (68) and also those mentioned by Koe [106].

CLINICAL EFFICACY AND STRUCTURE

As clinical effectiveness is a primary target in drug research those non-tricyclics giving pronounced clinical improvement in human depression are collected in *Table 6.2*. Side effects are omitted because often these are not related to the therapeutic effect.

Of almost all structural types mentioned above, representatives are present and structural conclusions, beyond those in the preceding sections are difficult to draw. A thorough structural analysis for more detailed interrelations would, however, be worthwhile.

Yet it should be noted that a small number of clinically effective compounds do not meet the simple structural requirements already indicated, a fact that may lead to the conclusion that different interaction areas can exist for the treatment of depression, areas in which neither NA nor 5-HT are the primary mediating factors, e.g., in the case of (96), (124).

Table 6.2. COMPOUNDS WITH THERAPEUTIC EFFICACY IN DEPRESSION

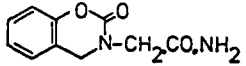
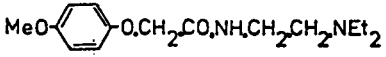
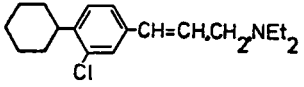
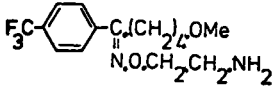
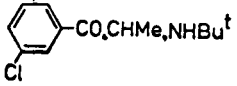
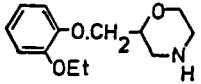
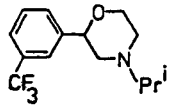
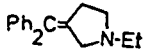
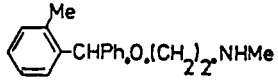
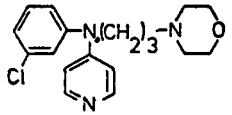
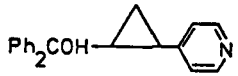
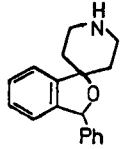
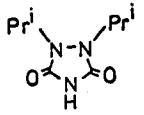
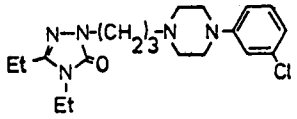
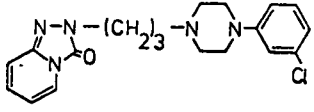
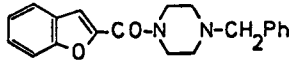
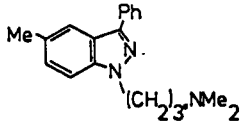
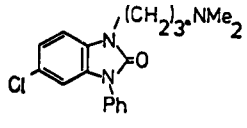
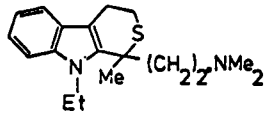
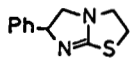
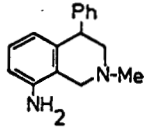
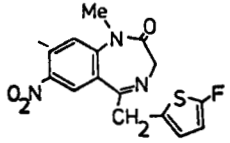
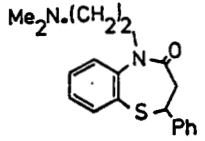
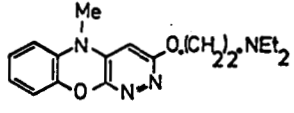
(No)	*Name*	*Formulae*	*Type of Depression* [a]	*Trial* [b]	*Mechanism* [c]	*References* [ ]
13	Caroxazone		Neurotic	C	MAOI	64–67
14	Mefexamide		Neurotic Reactive	O		70
41	933 CB		(Deep)	O [d]		114
42	Fluvoxamine		Retarded, endogenous	C	5-HT pref.	127
45	Bupropion		Endogenous (chronic)	C		129,130
46	Viloxazine		Anxious	C	MA	137

Table 6.2. COMPOUNDS WITH THERAPEUTIC EFFICACY IN DEPRESSION (continued)

(No)	Name	Formulae	Type of Depression [a]	Trial [b]	Mechanism [c]	References [ ]
51	Oxaflozane			O		149,150
66	AHR 1118		Retarded (severe)	C		170
69	Tofenacine		Neurotic and mixed	C	NA, 5-HT	182
73			Depressive neurosis	O		205
75	Cyprolidol		Endogenous	C		213
88	HP 505			O [d]	NA, 5-HT, DA	244

96	A 10749			C		258–261
97	ST 1191		Depressive neurosis	C		262
98	Trazodone			C	5-HT pref.	265
100	DIV 154			O [d]		267
105	Daledalin			O [d]		271–273
107	Clodazone		Retarded	O		277–279
109	Tandamine			O [d]	NA pref.	288

124	Tetramisole Dexamisole		Schizophrenic	C		306,307
130	Nomifensine		(Unipolar)	O	NA and DA pref.	327
137	LA-XIV		(Deep)	O		334
138	Thiazesim		Endogenous Retarded	O		335–338
143			(Mild to moderate)	O		348

[a] Type (and degree) of depression in which the compound was found effective.

[b] Performance of the clinical trial(s): C = controlled and O = open trial.

[c] The mechanism is mentioned only for those compounds of which a clear influence on the monoamine (MA) transmission is described to which the therapeutic effect may be ascribed: the reuptake inhibition of NA, DA and/or 5-HT or a (reversible, selective) MAO inhibition. When the reuptake mechanism of some of the MA is preferred, this is indicated with pref.

[d] 'Propably' open trial.

CONCLUSIONS

(1) In the non-tricyclic antidepressant structures there are promising drugs in which the side effects of the tricyclics are absent (*Table 6.2*). How far other serious side effects will limit their use will be disclosed by future studies, but surely some compounds will survive the careful clinical examinations.
(2) Probably in future, drugs with different profiles may appear for the various forms and symptoms of depression. Selectivity concerning elevation of mood, in contrast to physical stimulation, is reported for some compounds, e.g. (27). Also differences in amine metabolism between patients may give rise to a more sophisticated approach in pharmacotherapy.
(3) One of the clinically most desirable features of antidepressants to be developed might be a more rapid onset of action. No pharmacological approach aiming at this goal has as yet been described. Almost no clear clinical proof of improvement has in this respect come forth so far.
(4) A relation between selective 5-HT uptake inhibition *in vivo* and selective clinical mood-elevation has not been published hitherto. This may be due to a lack of correlation between current diagnostic techniques in psychiatry. Moreover most of the relevant selective inhibitors are just at the beginning of clinical investigations.
(5) Some compounds which are without any effect on NA or 5-HT uptake inhibition show therapeutic effectiveness. This may in the future lead to proposals for quite different biochemical mechanism of action in depression.
(6) Of the circa 140 collected structures, 55 have been tested in humans (47 in patients), resulting in circa 24 with clear therapeutic effectiveness. Thus there must be some relation between human depression and the pharmacological tests used. It is remarkable, however, that of only 7 of this 24 an effect on re-uptake mechanism have been mentioned.
(7) Compounds which proved very active in the well known reserpine and/or tetrabenazine antagonizing tests are sometimes found to be of low activity or inactive in clinical practice however e.g. (31), (52). This illustrates the well known fact of the poor correlation between existing animal experiments and clinical efficacy in human depression: research for developing more reliable animal tests is continuing, e.g. [7].

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INDEX OF COMPOUND NAMES AND CODE NUMBERS, COMPANIES AND PHASE OF TESTING

Name [a]	*No* [b]	*Year* [c]	*Company* [d]	*Test* [e]
933 CB	41	1975	CLIN-MIDY	P
A 10749	96	1967	ABBOTT	P
A 25794	9	1971	ABBOTT	P
Alaproclate	7	1976	ASTRA	P
Aletamine	27	1972	NAT. DRUG.	P
Amedaline	104	1969	PFIZER	P
AHR 1118	66	1970	ROBINS	P
AY 23673	113	1975	AYERST	A
AY 24614	111	1976	AYERST	A
BRL 14342	78	1977	BEECHAM	H
Bupropion	45	1974	BURROUGHS WELLCOME	P
BW 247	65	1975	BURROUGHS WELLCOME	H
Caroxazone	13	1968	FARMITALIA	P
Citalopram	84	1977	LUNDBECK	H
Clodazone	107	1968	WANDER	P
Clovoxamine	43	1970	PHILIPS-DUPHAR	P
CP-24,441	32	1974	PFIZER	A
Cyclazodine	142	1970	STERLING	P
Cyprolidol	75	1965	NEISLER	P
Daledalin	105	1969	PFIZER	P
Dexamisol	124	1972	JANSSEN	P
DIV 154	100	1976	HOECHST	P
EN 3022	108	1971	ENDO	A
EXP 561	31	1967	DUPONT	P
Femoxitine	52	1975	FERROSAN	P
Fluoxetine	71	1974	LILLY	H
Fluvoxamine	42	1970	PHILIPS-DUPHAR	P
FS 32	106	1975	CHUGAI	H
HP 505	88	1975	HOECHST	P
HT 3261	89	1976	MERCK	P
ICI 53, 165	86	1972	ICI	A
Imafen	125	1975	JANSSEN	A
Incazan	119	1973	ORDZCHONIKIDSE	P
K 8409	144	1975	ERBA	P

INDEX OF COMPOUND NAMES AND CODE NUMBERS, COMPANIES AND PHASE OF TESTING (CONTINUED)

Name [a]	*No* [b]	*Year* [c]	*Company* [d]	*Test* [e]
L 7526	15	1975	LABAZ	P
LA-XIV	137	1964		P
LM 5005	102	1977	GROUPE PHARMUKA	A
LM 5008	103	1977	GROUPE PHARMUKA	A
LU 3-049	82	1966	LUNDBECK	A
LU 3-057α	83	1966	LUNDBECK	A
Medifoxamine	72	1971	GERDA	A
Mefexamide	14	1964	ANPHAR	P
N 1157	10	1966	RICHTER	A
Nisoxetine	70	1975	LILLY	P
Nomifensine	130	1971	HOECHST	P
ORG 6582	34	1976	ORGANON	A
Oxaflozane	51	1973	CERM	P
PF 82	79	1974	DAINIPPON	H
PF 257	17	1974	DAINIPPON	A
Pirandamine	112	1975	AYERST	A
Pyrovalerone	44	1976	WANDER	P
Quipazine	129	1971	MILES	H
SCH 12650	77	1969	SCHERING	A
ST 1191	97	1975	ANGELINI	P
Talopram	80	1966	LUNDBECK	A
Talsupram	81	1966	LUNDBECK	A
Tandamine	109	1975	AYERST	P
Tetramisole	124	1972	JANSSEN	P
Thiazesim	138	1963	SQUIBB	P
Thozalinone	90	1966	LEDERLE	P
Tifemoxone	49	1976	DELALANDE	P
Tofenacine	69	1971	BROCADES	P
Toloxatone	94	1973	DELALANDE	A
Trazodone	98	1974	ANGELINI FRANCESCO	P
U 23807A	74	1965	UPJOHN	P
Viloxazine	46	1972	ICI	P
WIN 27, 147-2	114	1972	WINTHROP	P
WY 23409	116a	1972	WYETH	A
Zimelidine	68	1976	ASTRA	P

[a] Code numbers are only mentioned when a name is not available.

[b] Numbers of formulae in the text of this review.

[c] First year on which publications appeared.

[d] Abbreviations are those used in The Merck Index 9th edition (1976), explained in its Company Register (MISC-37) section.

[e] H, the compound is tested in healthy humans; P, patients are also treated; A, only animal experiments were known at the closing date of the manuscript.

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7 The Chemistry and Biological Activity of α -(*N*)-Heterocyclic Carboxaldehyde Thiosemicarbazones

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INTRODUCTION

The α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones constitute a class of agents which possess both antineoplastic and antiviral activity [1]. The first agent of this series to be examined for biological activity, 2-formylpyridine thio-

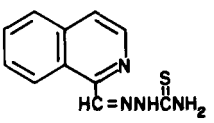
semicarbazone, was shown to possess mild antileukemic activity against the L-1210 tumour in mice at levels of drug which produced significant toxicity [2]. This finding, however, ultimately led to the synthesis of a variety of aromatic and heterocyclic carboxaldehyde thiosemicarbazones in an effort to develop more efficacious antineoplastic agents [3–6]. However, glyoxal bis(thiosemicarbazone) and analogues thereof were the only agents found to be inhibitory to Sarcoma 180 in mice [3]. Among these materials are the thiosemicarbazones of methylglyoxal and of 3-ethoxy-2-oxobutyraldehyde (ketoxal, Kethoxal), which have been shown to possess antineoplastic activity in murine systems; discussion of derivatives of this type are not included in this review. These studies led to the synthesis of a series of related bis(N^4-methylthiosemicarbazones) of diketones, dialdehydes and ketoaldehydes in which the carbonyl functions were separated by a variable number of methylene groups [7]. A series of thiosemicarbazones of oxypolysaccharides has also been reported [8].

French and Blanz [9–13] extended these observations by testing a series of formyl heteroaromatic thiosemicarbazones for anticancer activity; several of these derivatives, especially 1-formylisoquinoline thiosemicarbazone [10], 2-formyl-3-hydroxypyridine thiosemicarbazone [11] and 2-formyl-5-hydroxypyridine thiosemicarbazone [13] showed significant tumour-inhibitory activity when tested against a spectrum of transplanted murine neoplasms.

Initially, the clinical utility of these heterocyclic carboxaldehyde thiosemicarbazones as antineoplastic agents in man appeared to be limited by an inability to formulate them for parenteral administration because of their extreme water insolubility. Although 1-formylisoquinoline thiosemicarbazone has been shown to possess activity against transplanted tumours in mice when administered orally [10], the expectation of variable gastrointestinal absorption of this relatively water insoluble agent, as well as of related heterocyclic carboxaldehyde thiosemicarbazones, appeared to preclude oral usage as the ideal route of administration in clinical practice. A number of solvents, both polar and nonpolar, have been investigated in an effort to solubilize these drugs [14]; only dimethyl sulphoxide appeared to be capable of achieving the degree of solubilization necessary for the concentrations required in tests of antineoplastic potency. However, the use of dimethyl sulphoxide in man at present is questionable. Sodium salts have been prepared as a means of solubilizing these relatively insoluble compounds for parenteral administration [14]. In addition, a large number of structural modifications have been made to accomplish the insertion of hydrophilic groups such as NH_2 or OH in the heterocyclic ring systems, with the ultimate goal being the solubilization either as an acid salt or as a sodium salt, respectively.

HETEROCYCLIC SYSTEMS POSSESSING ANTINEOPLASTIC ACTIVITY

In 1966 French and Blanz [9] described a series of carboxaldehyde thiosemicarbazones of 16 different heterocyclic ring systems. These investigators studied various positions of attachment of the carboxaldehyde group relative to the ring hetero-atoms and concluded that the minimum requirement for biological activity was the attachment of the carboxaldehyde thiosemicarbazone side chain α to an unencumbered ring nitrogen of heteroaromatic character. Compounds in which the thiosemicarbazone side chain was attached at positions β or γ to the heterocyclic N-atom were inactive as antitumour agents. A conjugate N*-N*-S* tridentate ligand system was found to be a common feature of compounds with carcinostatic potency. For this reason, it was postulated that for anticancer activity: (a) the π-electron density at the point of attachment of the carboxaldehyde thiosemicarbazone side chain should be low, and (b) the ring nitrogen atom should be a reasonably good donor to transition metals to allow the formation of coordination compounds (chelates). Thus, quarternization of the ring nitrogen atom completely eliminated biological activity. A variety of heterocyclic ring systems carrying the thiosemicarbazone side chain α to the heteroaromatic nitrogen (1–7) are active antineoplastic agents. Thus, the portion of



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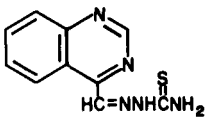

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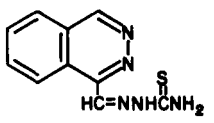

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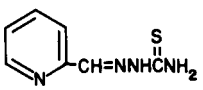

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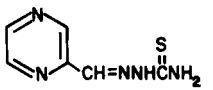

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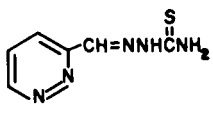

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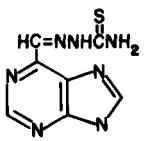


(7)

the molecule shown in *Figure 7.1* is a common feature of the active antineoplastic agents of this series except in compounds (3) and (6) in which the α' carbon atom is replaced by nitrogen. Removal of nitrogen from the ring system, i.e. in

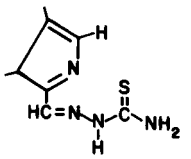
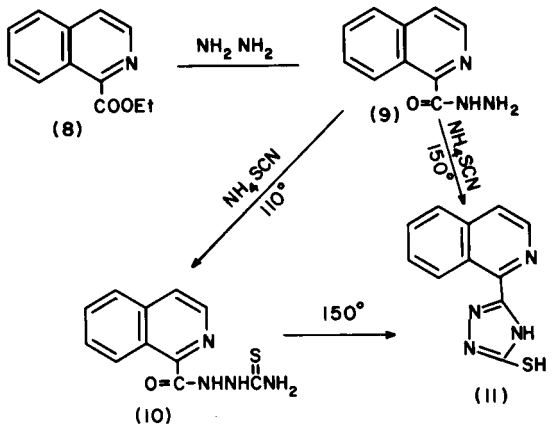


Figure 7.1. Structural parameters required for antitumour activity

aromatic aldehyde thiosemicarbazones such as naphthaldehyde thiosemicarbazone and salicylaldehyde thiosemicarbazone, has led to compounds with no anticancer activity. Pyridine and isoquinoline are the 2 heterocyclic ring systems which have been most extensively investigated for structure-activity relationships with this class of antineoplastic agents; structural modifications of these 2 ring systems will be described separately.

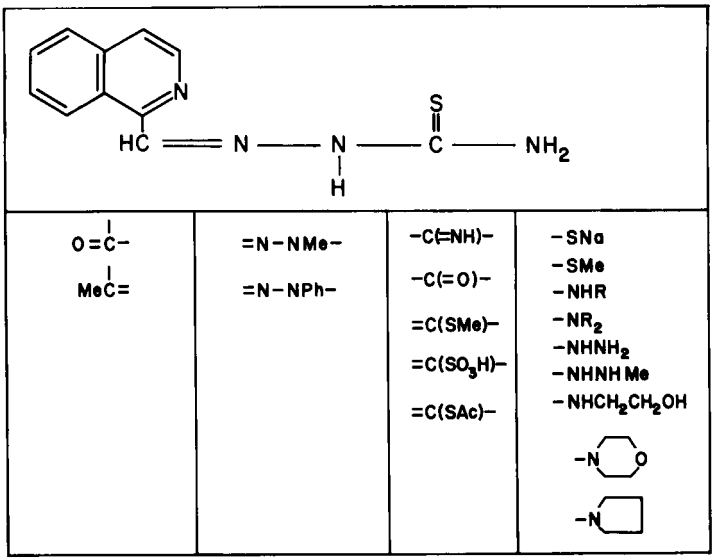
MODIFICATIONS OF THE THIOSEMICARBAZONE SIDE CHAIN

Agrawal and Sartorelli [15] have studied the effects of chemically modifying the side chain of the isoquinoline derivative of this class (1) on biological activity by fabricating a number of structures as shown in *Table 7.1.* The isoquinaldinoylthiosemicarbazide (10), in which the aldehyde hydrogen is replaced by a carbonyl oxygen, was obtained by treating ethyl isoquinaldinate (8) with

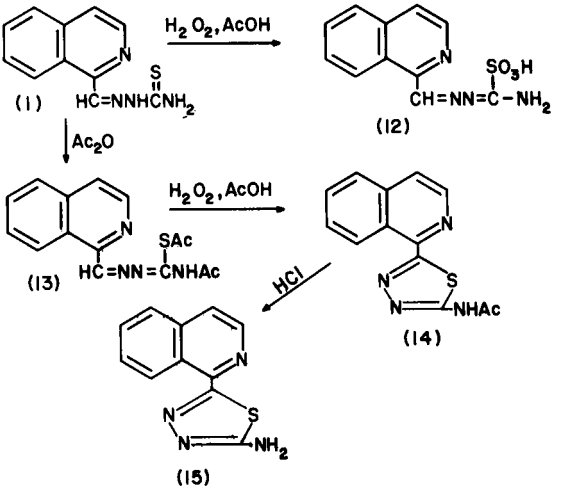


hydrazine to give isoquinaldinic acid hydrazide (9), followed by reaction with NH_4SCN at 110°. When the reaction temperature was raised to 150°, loss of

Table 7.1. VARIATIONS IN THE STRUCTURE OF 1-FORMYLISOQUINOLINE THIOSEMICARBAZONE [15]



1 mole of water and cyclization to 3-mercapto-5-(1-isoquinolyl)-1,2,4(H)-triazole (11) occurred.



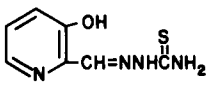
1-Amino-4-isoquinolyl-2,3-diazabuta-1,3-diene-1-sulphonic acid (12) was

obtained by oxidation of (1) with hydrogen peroxide and acetic acid. Acetylation of (1) produced 1-formylisoquinoline N^4,*S*-diacetylthiosemicarbazone (13) which, upon oxidation with hydrogen peroxide and acetic acid, cyclized the side chain to yield the thiadiazole derivative (14); acid hydrolysis produced the corresponding aminothiadiazole (15).

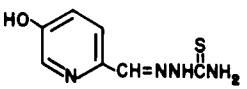
Substitution of the aldehyde hydrogen atom with either a methyl function or a carbonyl oxygen, or the proton on the 2′-nitrogen atom with a methyl or phenyl group, resulted in a loss of antineoplastic activity. Replacement of the sulphur atom with an =NH group caused a decrease in anticancer activity, and other changes in this position produced a complete loss of biological potency. Changes in the terminal thioamide portion of the molecule either markedly lessened or completely eliminated the capacity of (1) to retard the growth of mammalian cells. Furthermore, cyclization of the side chain, as in compounds (11), (14), and (15), also resulted in inactive compounds [15]. Substitution of methyl, ethyl, or phenyl groups for a hydrogen atom on the terminal nitrogen resulted in compounds in which the biological activity was reduced or lost [9].

MODIFICATIONS OF 2-FORMYLPYRIDINE THIOSEMICARBAZONE

The initial observation of substituent effects on the pyridine derivative (4) was demonstrated by the introduction of a hydroxyl group at the C-3 position to yield (16) [11]. This hydroxylation increased tumour-inhibitory activity significantly and at the same time reduced toxicity 25-fold. Formation of the ethyl ether of (16), however, diminished anticancer potency. The increased activity of the 3-hydroxy isomer prompted the synthesis of the 5-hydroxy derivative (17)



(16)



(17)

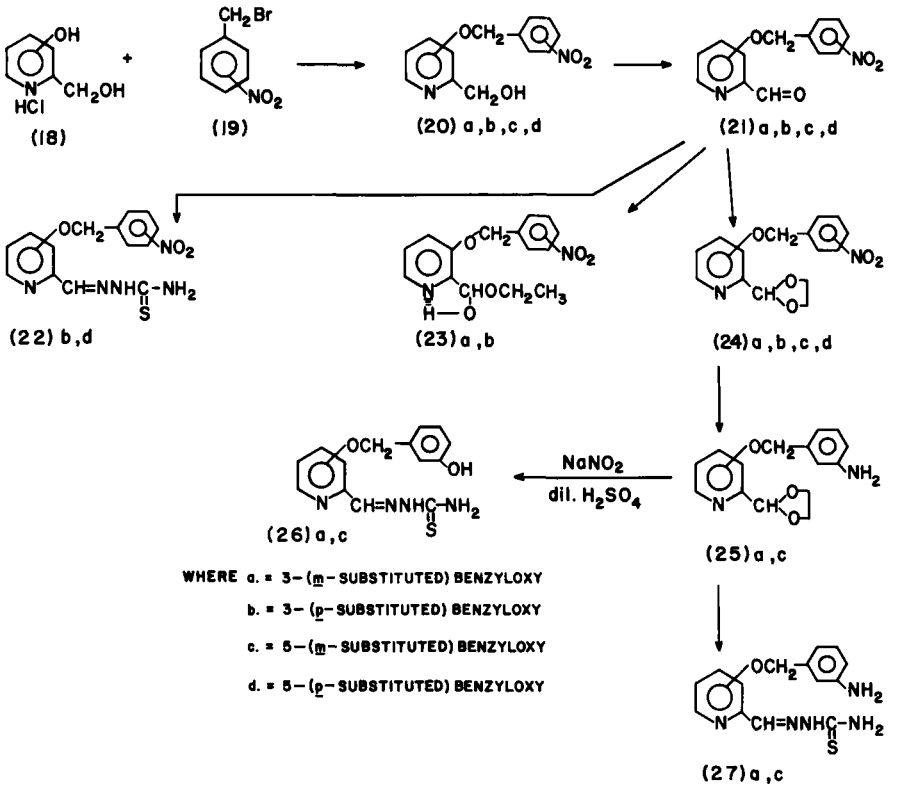
which was found to be one of the most active agents of this series [17].

Monomethylated derivatives of 2-formylpyridine thiosemicarbazone have been synthesized to define molecular dimensions compatible with antineoplastic activity. These were the thiosemicarbazones of 3-, 4-, 5-, and 6-methylpyridine-2-carboxaldehyde [16]. The 3-, 4-, and 5-methylated pyridine derivatives were found to be active tumour-inhibitory agents, indicating that substitution of such alkyl groups did not interfere with biological potency. However, introduction of the methyl group in the 6 position of the pyridine ring resulted in a compound with no antineoplastic activity, suggesting an apparent intolerance to substitu-

tion at the position α' to the heterocyclic nitrogen atom for growth inhibitory action [17,18].

A series of additional 3- and 5-substituted derivatives of 2-formylpyridine thiosemicarbazone have been synthesized [19] in which the 3-substituent was COOH or F and the 5-substituent was F, Cl, Br, I, Et, OCF_3, CF_3, $N(Me_2)$, O_2SMe, and OAc. The effect of these substituents on antitumour activity was tested in several systems; however, potency against a particular tumour followed no simple parametric rules. Furthermore, the order of substituent effects changed markedly from one tumour system to another [19].

A series of meta and para substituted 3- and 5-benzyloxy derivatives of 2-formylpyridine thiosemicarbazone has been synthesized both to explore and to utilize a hypothetical hydrophobic binding zone adjacent to the major inhibitor binding site of the target enzyme, ribonucleoside diphosphate reductase [20]. These agents were synthesized by condensing a nitrobenzyl bromide (19) with 3- or 5-hydroxy-2-hydroxymethylpyridine (18), followed by oxidation with MnO_2

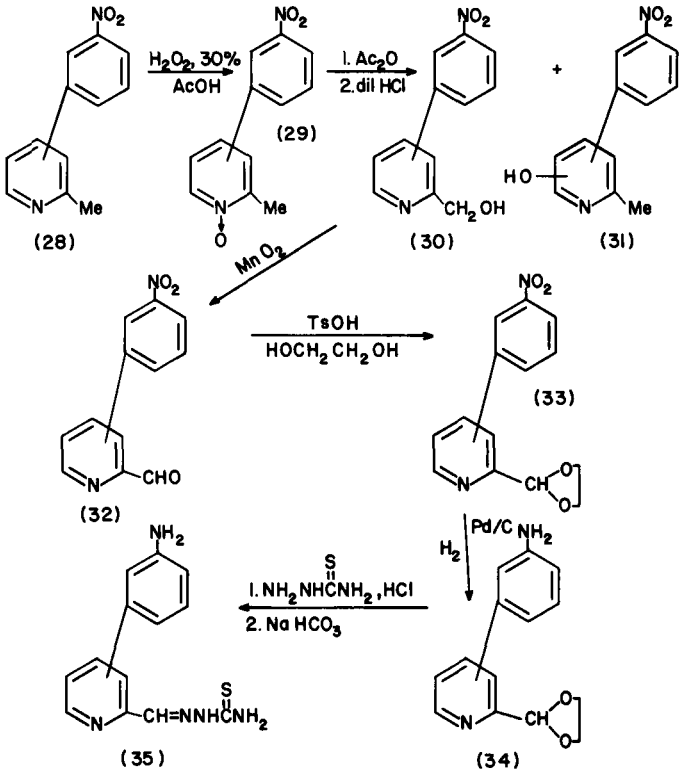


(27) a,c

to yield corresponding aldehydes (21), which were derivatized to thiosemicarbazones (22) [20]. Formation of stable hemiacetals (23a, 23b) was demonstrated when (21a) and (21b), respectively, were recrystallized from ethanol. The hemiacetal OH group formed a strong intramolecular hydrogen bond (3180 cm^{-1}, KBr). Interestingly, the corresponding 5-isomers (23c) and (23d) failed to form stable hemiacetals with ethanol under similar conditions. Although stable cyclic hemiacetals are rather common among carbohydrates and steroids, there are relatively few examples of stable non-cyclic hemiacetals [21]. The NMR spectrum in $CDCl_3$ indicated that an equilibrium between free aldehyde and hemiacetal existed in approximately a 1 : 1 ratio. Catalytic hydrogenation of m-nitrobenzyloxypyridine (24a) and (24c), using 5% Rh-carbon as the catalyst under 10 lb $inch^{-2}$ pressure, gave a good yield of the corresponding amines (25a) and (25c); the para isomers (24b) and (24d) failed to give the desired amines under the same conditions. However, treatment of (20a–d) with iron powder and acetic acid in the presence of acetic anhydride at 60° gave corresponding diacetylated derivatives in high yield. When the reduction was carried out in the absence of acetic anhydride, the *p*-amino group participated in the cleavage of the benzylic ether linkage. The formation of NHAc decreased the availability of the free electron pair on the N atom and therefore increased the stability of the ether bond.

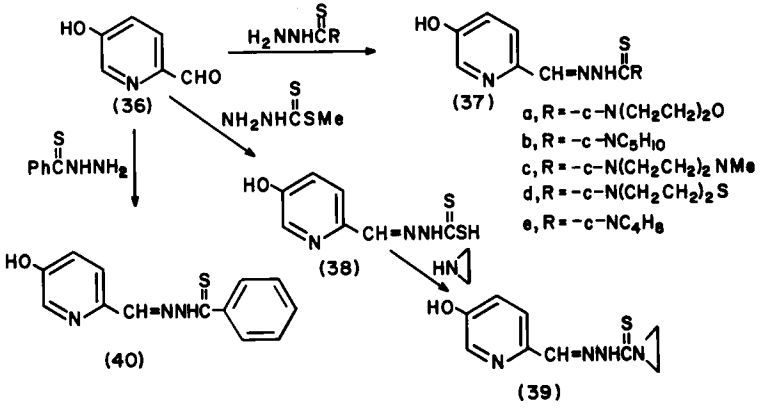
Several *m*-nitro- and *m*-aminophenyl-substituted 2-formylpyridine thiosemicarbazones have been synthesized [22]. *m*-Nitrophenyl-2-picolines were formed by coupling 2-picoline with diazotized *m*-nitroaniline. The 3-, 4-, 5-, and 6-substituted *m*-nitrophenyl-2-picolines were separated by fractional crystallization and their structures confirmed by NMR. These derivatives were then converted to N-oxides (29) and allowed to react with acetic anhydride to form corresponding 2-methyl acetates. Acid hydrolysis of esters produced carbinols (30) which were oxidized with manganese dioxide to carboxaldehydes (32) and then converted to thiosemicarbazones. Smaller amounts of phenolic esters with an acetoxy group in the pyridine ring were also formed during the rearrangement of the N-oxides; these were hydrolyzed to phenolic compounds. *m*-Nitrophenyl-2-picolinaldehydes (32) were converted to cyclic ethylene acetals (33), which were subsequently reduced by catalytic hydrogenation to yield corresponding amino derivatives (34). 2-Formyl-4-(3-aminophenyl)pyridine thiosemicarbazone was the most active antineoplastic agent of this series in mice bearing Sarcoma 180 ascites cells.

A series of 4′-substituted derivatives of 5-hydroxy-2-formylpyridine thiosemicarbazone (17) has been synthesized and evaluated as inhibitors of alkaline phosphatase activity of a murine ascitic cell line of Sarcoma 180 resistant to the antileukemic agents, 6-mercaptopurine and 6-thioguanine [23]. These agents were also tested as inhibitors of ribonucleoside diphosphate reductase from the

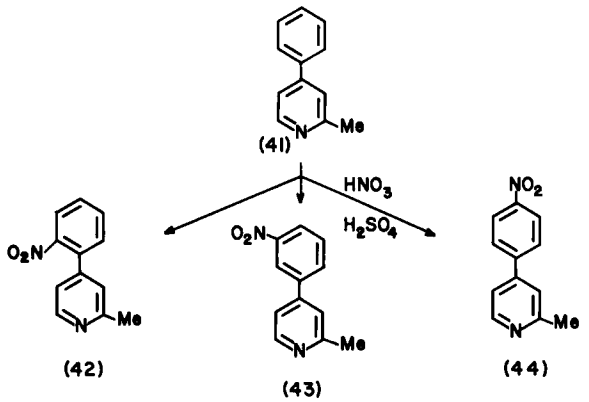


Novikoff hepatoma of the rat and for antineoplastic activity in mice bearing either 6-thiopurine-sensitive or -resistant cells of Sarcoma 180. Structure-activity relationship studies have delineated the bulk requirement for a five-membered ring at 4′-position for optimum alkaline phosphatase-inhibitor interaction. The presence of this bulk resulted in loss of inhibitory activity by α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones for ribonucleoside diphosphate reductase. However, some of these agents were found to have retained tumour-inhibitory activity. 4′-Substituted thiosemicarbazides, the intermediates employed for the syntheses of corresponding thiosemicarbazones, were prepared by the procedures of McElhinney [24], except that reaction of ethyleneimine was not successful with methyl dithiocarbazate; therefore, the ethyleneimine derivative (39) was obtained first by forming the corresponding methyl dithiocarbazate ester (38) and then by reacting it with ethyleneimine.

Since 2-formyl-4-(3-aminophenyl)pyridine thiosemicarbazone was found to be an active antineoplastic agent [22], two other isomers have been synthesized in which the amino group was inserted at either the ortho or para positions of



the 4-phenyl moiety [25]. Syntheses of these derivatives were achieved by nitration of 4-phenyl-2-picoline (41), which produced a mixture of *o*-, *m*-, and *p*-nitro-substituted derivatives (42–44). These isomers were separated by the



solubility differences of their hydrochloride or nitrate salts in 10, 27, and 40% yields, respectively. Identification and confirmation of the structures of these isomers were carried out by NMR. Each isomer was individually subjected to a series of reactions to oxidize the 2-Me group to the corresponding carboxaldehyde and to reduce the nitro function to an amino group. These agents were tested for antineoplastic activity in mice bearing Sarcoma 180 ascites cells; both the *o*- and *p*-amino-substituted derivatives were found to be inactive. The inactivity of the ortho- and para-substituted amino derivatives can possibly be explained by the mesomeric stabilization of the cationic species, as shown in *Figure 7.2* for

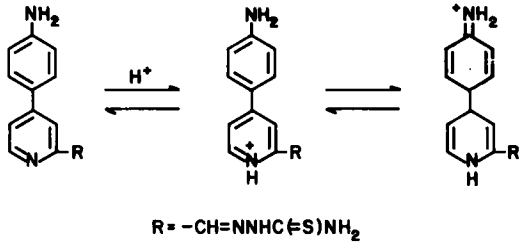
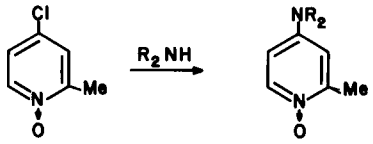


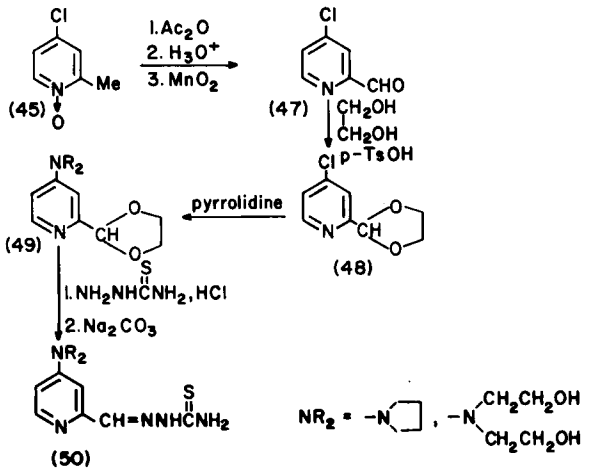
Figure 7.2. Resonance forms of 2-formyl-4-(4-aminophenyl)pyridine thiosemicarbazone

the para substituent. Similar structures can also be formed with the ortho substituted compound. Formation of these species tends to hinder the coordination with metals of a pair of free electrons at the ring nitrogen. Since evidence is available that such coordination of the ring nitrogen with iron might be involved in the tumour-inhibitory action of this class of agents [26], the formation of cationic species appears to explain inactivity.

A series of 4-substituted 2-formylpyridine thiosemicarbazones have also been synthesized [27] in which a tertiary nitrogen atom, as part of different ring systems, was inserted to achieve the assets of amino-substituted derivatives while (a) minimizing enzymatic conjugation of the nitrogen atom employed for solubilization and (b) utilizing the postulated hydrophobic binding zone present in or adjacent to the inhibitor binding site on the target ribonucleoside diphosphate reductase enzyme by varying the size of the substituted ring. Synthesis of these compounds was carried out by reacting 4-chloro-2-picoline N-oxide (45) with various secondary amines to produce 4-substituted derivatives (46). The amines utilized in this reaction were dimethylamine, morpholine, *N*-methylpiperazine, piperidine, and imidazole. Selection of these amines was based on their basicities, in an effort to cover a relatively wide range of pK_a values. Oxidation of the 2-Me group to the aldehyde was carried out by treatment of 4-



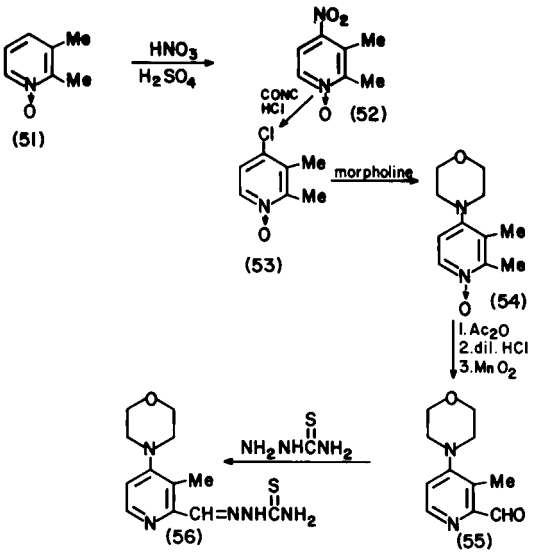
substituted 2-picoline N-oxides with excess acetic anhydride to yield corresponding acetoxymethyl derivatives, which upon either acid or base hydrolysis produced 2-carbinols. These materials were oxidized further with manganese



dioxide to yield the corresponding 2-carboxaldehydes, which upon reaction with thiosemicarbazide resulted in the desired thiosemicarbazones. However, the introduction of a pyrrolidine group in this reaction sequence resulted in a considerable amount of polymerization. Therefore, an alternative procedure was employed; this included protection of 4-chloro-2-formylpyridine (47) by formation of an ethylene ketal (48), which upon nucleophilic substitution with pyrrolidine produced the corresponding pyrrolidine ketal (49). Reaction of the ketal (49) with thiosemicarbazide in the presence of hydrochloric acid produced the 4-pyrrolidino derivative (50). A similar procedure was employed to introduce a diethanolamine group.

4-Morpholino-2-formylpyridine thiosemicarbazone was the most active antineoplastic agent of this series in mice bearing Sarcoma 180 ascites cells and was significantly superior to the 5-hydroxy analogue (17) in this test system. Insertion of a 3-Me group onto the morpholino analogue was also carried out (a) to determine the steric influence of this substituent on the properties of the 4-morpholino derivative and (b) to enhance interaction of the inhibitor with the hydrophobic bonding zone of the target enzyme, ribonucleoside diphosphate reductase. Synthesis was achieved by utilizing the sequence of reactions in which 2,3-dimethylpyridine N-oxide was converted to the 4-chloro derivative via formation of a 4-nitro analogue (52) [28], utilizing a procedure similar to that employed for the formation of 4-chloro-2-picoline N-oxide [29]. The 4-chloro derivative (53) was then transformed through a series of reactions to the 2-carboxaldehyde (55).

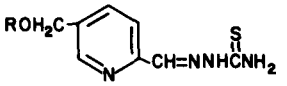
Compound (56) was found to be less active against Sarcoma 180 than was the morpholino derivative without a 3-methyl group [27]. This finding was un-



expected since earlier results had demonstrated that the insertion of the hydrophobic Me group onto the 3-position of the pyridine ring system increased the affinity of the inhibitor for the target enzyme, ribonucleoside diphosphate reductase [30]. A possible explanation for the relatively low anticancer activity of (56) is that the 3-Me group of (56) forces the 4-morpholino substituent and the pyridine ring out of coplanarity; this explanation requires the necessity of a coplanar molecule for the biological activity of this class of compounds.

The morpholino group has also been inserted in place of the terminal amino group of the side chain in both the 3- and 5-hydroxypyridine derivatives (16,17); such substitution has been found to result in more host toxicity and less tumour-inhibitory activity than that possessed by the parent compounds [31].

An extensive presentation of biological data produced by a total of 33 pyridine aldehyde thiosemicarbazones has been described [32]. This report also included data on 26 isoquinoline carboxaldehyde thiosemicarbazones and 45 other heterocyclic aldehyde thiosemicarbazones. However, no attempt was made to correlate structure with activity. Recently, a series of 5-substituted analogues of (4) has been reported [33] which included various esters of 5-hydroxymethyl-pyridine thiosemicarbazone. 5-Acetoxymethyl (57b) and 5-isonicotinoyl (57f) derivatives were found to be active antineoplastic agents. The acetoxymethyl derivative (57b) was markedly active against the Ehrlich ascites carcinoma, leukemia L-1210, and leukemia C-1498, while moderately effective against Nakahara–Fukuoka sarcoma and inactive against adenocarcinoma 755. The iso-



(57)

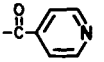
a. R= H

b. R= -C(=O)-Me

c. R= -C(=O)-Ph

d. R= -C(=O)C$_6$H$_4$ (p-Cl)

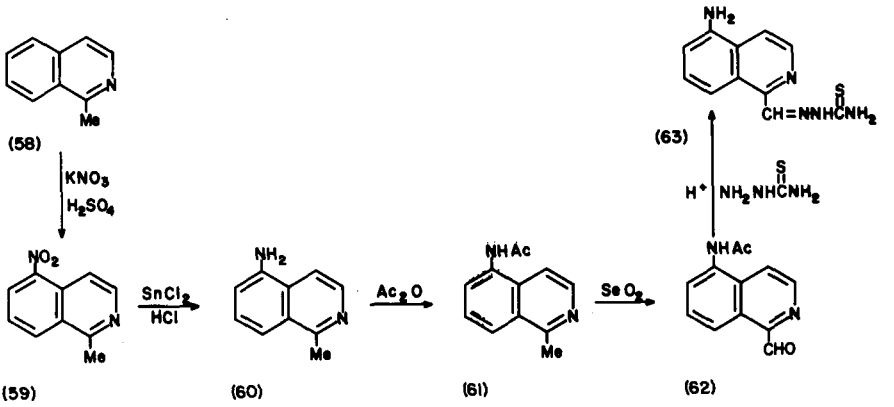
e. R= -C(=O)C$_6$H$_4$ (p-NO$_2$)

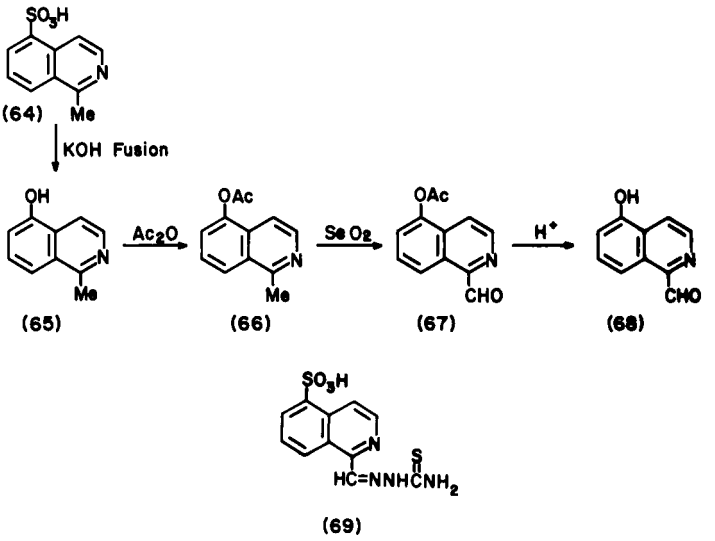
f. R= -C(=O)- 

nicotinoyl derivative (57f) was also markedly active against the Ehrlich ascites carcinoma, leukemia L-1210, and leukemia C-1498, moderately active against adenocarcinoma 755, and slightly active against Nakahara–Fukuoka sarcoma.

MODIFICATIONS OF 1-FORMYLISOQUINOLINE THIOSEMICARBAZONE

The first systematic approach to evaluate the effects of various substituents on the heterocyclic ring on the biological activity of these agents was undertaken by Agrawal, Booth and Sartorelli [34]. The candidate deemed most appropriate for such a study was 1-formylisoquinoline thiosemicarbazone (1), since it was extensively employed in biochemical studies as the model agent of the series and had been shown to cause pronounced inhibition of the growth of a relatively



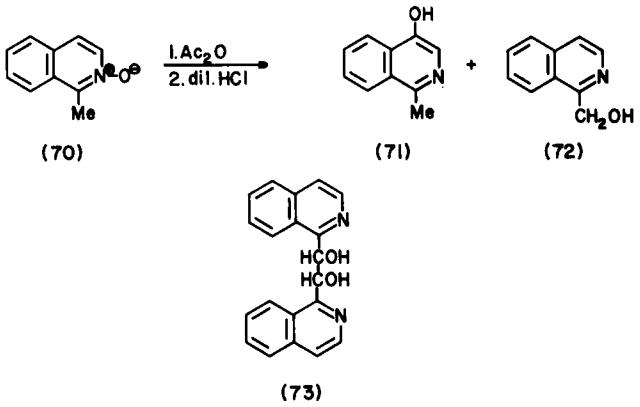


wide spectrum of transplanted rodent tumours [10] and to cause tumour regression in spontaneous lymphomas of dogs [35,36].

The initial approach to evaluate substituent effects involved the synthesis of a series of 5-substituted derivatives of (1) [34]. Nitration of 1-methylisoquinoline (58) produced only one isomer (59), which was reduced to give the amino analogue (60). Acetylation of (60), followed by oxidation, acid hydrolysis and reaction with thiosemicarbazide produced the corresponding thiosemicarbazone (63). 1-Methylisoquinoline (58) was also sulphonated at the 5-position to yield (64) which upon alkali fusion formed the 5-hydroxy compound (65). Acetylation of (65), followed by oxidation and acid hydrolysis produced the corresponding 5-hydroxy aldehyde (68). 1-Formylisoquinoline thiosemicarbazone (1) was also sulphonated directly at room temperature using fuming H_2SO_4 to give (69). This compound has also been synthesized by the Krohnke method via a pyridinium salt and nitrone intermediate [37]. Evaluation of the biological potency of these 5-substituted derivatives indicated that such substitution reduced carcinostatic activity in the case of the NO_2, NHAc, and SO_3H derivatives, whereas the insertion of NH_2, OH, or OAc groups resulted in derivatives possessing tumour-inhibitory activity comparable with that of the parent compound (1). Furthermore, the substitution of OH and OAc in the 5-position of the isoquinoline ring system of (1) yielded derivatives that were considerably less toxic to the host than the parent compound (1). The findings which indicate that substitution of electron-withdrawing groups at the 5-position of (1) results in a decrease in tumour-inhibitory potency do not appear to be consistent with

the postulation of French and Blanz [9] that a low π-electron density at the point of attachment of the aldehyde moiety is required for carcinostatic activity, since the substitution of electron-withdrawing groups would be expected to decrease the π-electron density at the 1-position. Furthermore, the insertion in position 5 of (1) of electron-donating groups such as amino or hydroxy resulted in derivatives capable of prolonging the life span of tumour-bearing animals to the same extent as that produced by the parent compound.

The 2 substituents, OH and OAc, that increased therapeutic potency when substituted at the 5-position of (1), also have been introduced at the 4-position [38]. Position 4, being para to position 1, was considered to be of prime importance with regard to the exertion of electronic effects by the substituent group on the thiosemicarbazone side chain. Syntheses were accomplished by rearrangement of 1-methylisoquinoline N-oxide (70) with Ac_2O; acid hydrolysis resulted

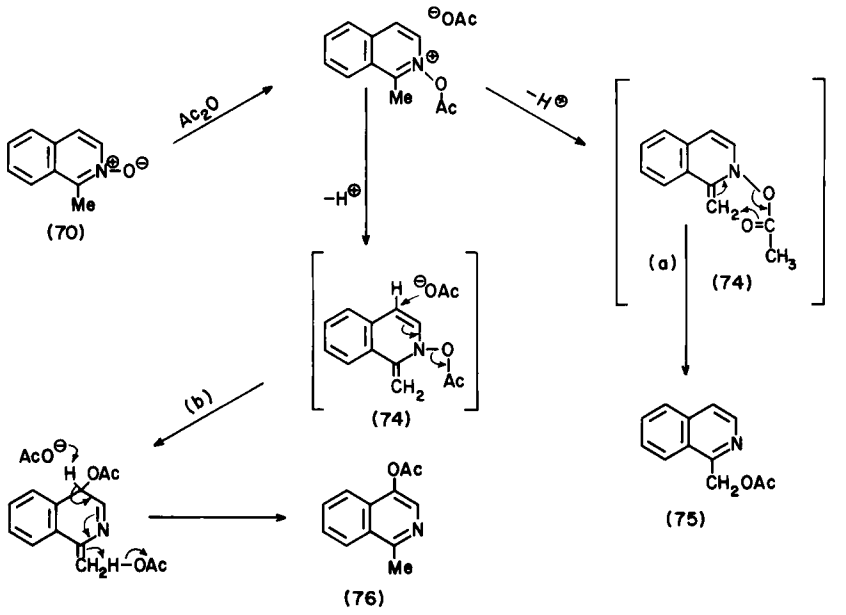


(70) (71) (72)

(73)

in 2 major products, 4-hydroxy-1-methylisoquinoline (71) and 1-hydroxymethylisoquinoline (72). A third compound was isolated in relatively small yield and was characterized by NMR and mass spectroscopy to be α,α′-bis(1-isoquinolyl)ethylene glycol (73).

Several possible mechanisms including ionic [39] and free radical [40] formation have been proposed for the analogous rearrangement of 2-picoline N-oxide. In the case of the isoquinoline series, where no rearrangement products at position 3 were isolated, an intermediate (74) resulting from abstraction of an active H from the 1-methyl by AcO^- would be reasonably expected to predominate in the transformations. Such an intermediate cannot be formed from 3-methylisoquinoline since it would result in the disruption of the aromaticity of the benzene ring. Conversion of (74) to the 1-acetoxymethyl derivative (75, pathway a) may result from an intramolecular rearrangement, analo-



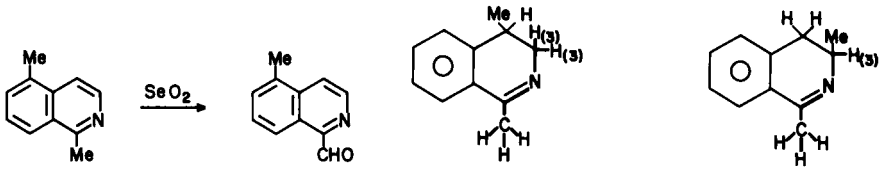
gous to that suggested for 2-picoline N-oxide [41]. Rearrangement to the 4-position of the isoquinoline ring most probably occurs by a series of addition and elimination reactions (pathway b) to produce 1-methyl-4-acetoxyisoquinoline (76).

Oxidation of 4-acetoxy-1-methylisoquinoline (76) with selenium dioxide produced the corresponding 1-carboxaldehyde, which upon acid hydrolysis yielded the 4-hydroxy derivative. Treatment of heterocyclic aldehydes with thiosemicarbazide produced the desired thiosemicarbazones. In general, the 4-substituted derivatives were less effective than (1) as antineoplastic agents against Sarcoma 180 ascites cells in mice; however, the sodium salt of the 4-hydroxy derivative was considerably more efficacious than (1) against the L-1210 lymphoma.

Thiosemicarbazones of 3-, 4-, and 5-methylisoquinoline-1-carboxaldehyde have also been synthesized [18] to assist in a definition of molecular dimensions compatible with biological activity. The syntheses of the methyl-substituted derivatives were accomplished by rearrangement of the N-oxides of 1,3-, and 1,4-dimethylisoquinoline with acetic anhydride followed by acid hydrolysis of the ester and subsequent oxidation of the carbinol with manganese dioxide. However, 1,5-dimethylisoquinoline (77) was selectively oxidized with selenium dioxide to the corresponding 1-carboxaldehyde (78) [18].

From the relatively large number of compounds investigated, some useful

empirical results have been obtained which can be used to characterize compounds in the isoquinoline series. As an example, unless C-8 or the ring nitrogen atom contains substituents (e.g. N → O), the chemical shift for a 1-Me group



(77) (78) (79) (80)

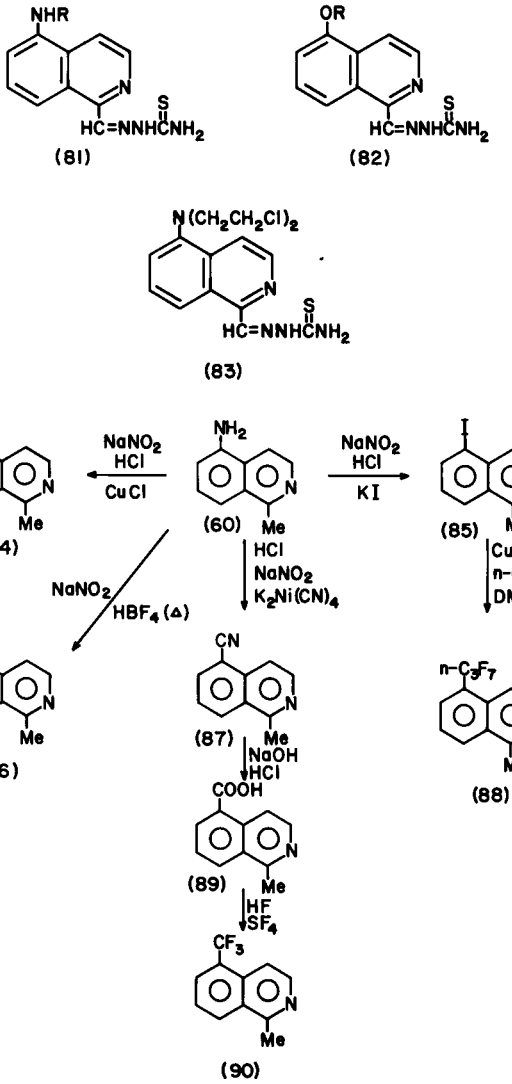
occurs between δ 2.81 and 2.92. Similarly, doubly bonded electronegative substituents on the carbon atom directly bonded to C-1 cause a paramagnetic shift of the proton resonance at C-8 into the region δ 9.13–9.26. This C-8 proton multiplet lies at lower field than do the other ring protons.

An interesting long-range coupling in isomers (79) and (80) of 3,4-dihydroisoquinolines was used to establish the cyclized nature of the compounds. In (79), the 1-Me substituent (δ 2.32) is a triplet, ^{J}1-Me,H-3 = 1.5 Hz, due to long-range coupling to the 2 H-3's (thick lines). On the other hand, in (80) the Me is a doublet showing a 5-bond long-range coupling, ^{J}1-Me,H-3 = 1.9 Hz, due to coupling with the single proton on C-3. Such long-range coupling must involve the heterocyclic nitrogen atom. Double irradiation of the 1-Me resonance lines in (79) reduced the multiplet structure at δ 3.42 to an octet which is the AB subspectrum of the $ABMX_3$ spin system.

The methyl substituted analogues were tested as inhibitors of the target enzyme ribonucleoside diphosphate reductase *in vitro*. The 3-methyl derivative was considerably less active as an inhibitor of enzyme activity than the parent compound (1). Similarly, the 6-methyl derivative of the pyridine analogue (4) was a very weak inhibitor. In contrast, however, 4- or 5-methyl substituted isoquinolines as well as 3-, 4-, or 5-methyl substituted 2-formylpyridine thiosemicarbazones were equal or better as inhibitors of ribonucleoside diphosphate reductase than the corresponding parent compounds. These studies led to the postulation that the decreased inhibitory potencies of the 3-methyl derivative of (1) and the 6-methyl derivative of (4) are expressive of low bulk tolerance by the enzyme to substituent groups in these analogous positions with respect to orientation at the inhibitor binding site [30].

Several groups potentially capable of alkylation of the enzyme, ribonucleoside diphosphate reductase, were introduced at the 5-position of the isoquinoline nucleus of (1) in an effort to design possible irreversible inhibitors of the enzyme [42]. Two series of compounds were made using either 5-amino or 5-hydroxy derivatives as precursors of corresponding amides (81) [-$NHSO_2Me$,

-$NHCOC_6H_4$ (*m*- or *p*-SO_2F)] or esters (82) [-OSO_2Me, -OCO_2Et, -OCO_2Ph, -$OCOC_6H_4$ (*m*- or *p*-SO_2F), -$OSO_2C_6H_4$ (*o*-, *m*-, or *p*-SO_2F)]. In addition, a bis(β-

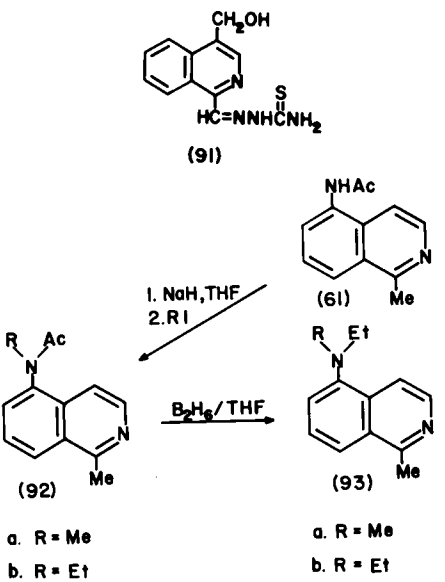


chloroethyl)amino group (83) was introduced at the 5-position by nucleophilic substitution of 5-chloro-1-formylisoquinoline. Although these agents were potent inhibitors of the enzyme *in vitro*, requiring a concentration in the range

of 10^{-8} to 10^{-6} M for 50% inhibition of the enzyme, only 2 derivatives, containing either a $-OCO_2Et$ or a $-OCO_2Ph$ moiety, were shown to have potent tumour-inhibitory activity in mice bearing Sarcoma 180 ascites cells [42].

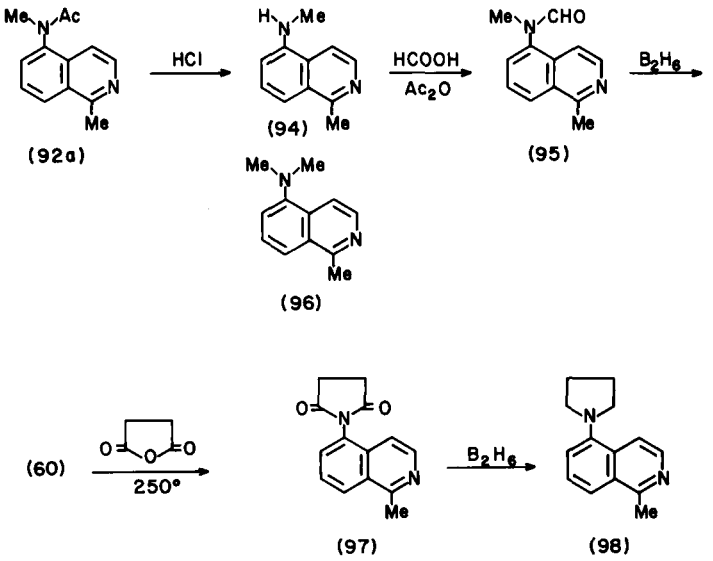
A series of additional substituents on the isoquinoline molecule (1) have been reported [37] and these have included the 2-oxide; 5-substituted derivatives (CN, Cl, F, CF_3, n-C_3F_7, COOH); 6-Me; 7-substituted derivatives (OAc, F, Cl, OH, OMe); and 8-F. 5-Substituted analogues were synthesized utilizing 1-methyl-5-aminoisoquinoline, which was converted by suitable diazotization reactions into (84, 85, 86, and 87). The iodine in compound (85) was replaced by reaction with n-C_3F_7I in the presence of activated Cu to yield (88). The nitrile was hydrolyzed to give the carboxylic acid (89) which was replaced with CF_3 by reaction with SF_4 in hydrofluoric acid. Oxidation of the substituted 1-methyl-isoquinolines with selenium dioxide produced the corresponding 1-carboxaldehydes which were converted to thiosemicarbazone derivatives. The biological activity of these agents varied depending upon the tumour system employed. No simple correlation of biological results with electronic, hydrophobic or hydrophilic parameters was obtained from these derivatives. A complicating factor in attempting to correlate *in vivo* antitumour activity with chemical structure is the poor absorption of these water-insoluble compounds; however, better correlations have been accomplished by measuring the degree of inhibition of the target enzyme by dimethyl sulfoxide solubilized material [30].

A 4-hydroxymethyl derivative (91) has been recently reported [33] to be



more active than the parent compound (1), but it was found to be relatively more toxic.

In an attempt to exploit the postulated hydrophobic bonding region at the inhibitor binding site of ribonucleoside diphosphate reductase, several 5-substituted monoalkylamino, dialkylamino, and *N*-alkylacetamido derivatives of 1-formylisoquinoline thiosemicarbazone were prepared [43]. Alkylation of the 5-amino derivative by an alkyl iodide in tetrahydrofuran or dimethylformamide using sodium hydride as a base afforded only small amounts of the desired alkylamino derivatives. However, alkylation was readily accomplished by initially activating the amino function by acetylation (61) and then reacting it with sodium hydride in anhydrous tetrahydrofuran; this procedure was followed by the necessary alkyl iodide to give (92) which was reduced by diborane in tetrahydrofuran to yield the corresponding methyl ethyl (93a) or diethyl (93b) analogues. The 5-*N*,*N*-dimethylamino derivative was prepared by first formylating the 5-methylamino-1-methylisoquinoline (94) obtained from the acid hydrolysis of the corresponding *N*-acetamido derivative (92a), and then by reducing the *N*-formyl derivative (95) with diborane to produce the dimethyl analogue (96). Fusion of 5-amino-1-methylisoquinoline (60) with succinic

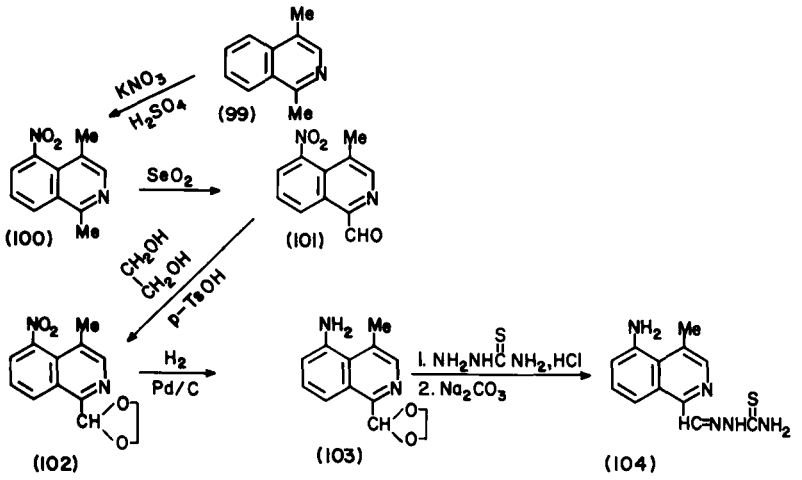


anhydride at 250° yielded the succinimido derivative (97) which was reduced with diborane to the pyrrolidinyl derivative (98). The 5-substituted 1-methyl isoquinolines were in general oxidized with selenium dioxide to produce the corre-

sponding 1-carboxaldehydes which were then condensed with thiosemicarbazide to yield the respective thiosemicarbazones.

Two of the derivatives (i.e., those containing 5-methylamino and 5-ethylamino groups) demonstrated impressive antitumour activity against Sarcoma 180 ascites cells and several others were potent inhibitors of ribonucleoside diphosphate reductase, requiring concentrations in the range of 10^{-8} to 10^{-6} M for 50% inhibition [43]. 5-Methylamino-1-formylisoquinoline thiosemicarbazone, which was the most effective of the newly synthesized compounds required a concentration of $3 \cdot 10^{-8}$ M for 50% inhibition of reductase activity and increased the life span of tumour-bearing mice over untreated animals by a factor of 2.5 at the optimal daily dose.

4-Methyl-5-amino-1-formylisoquinoline thiosemicarbazone was synthesized recently [44] in an attempt to obtain a compound of this class with (a) high affinity for ribonucleoside diphosphate reductase, (b) water solubility as an acid salt of the amine, (c) steric protection of the amino group from *in vivo* acetylation, and (d) insensitivity to O-glucuronidation, a major factor in the inactivity as an anticancer agent in man of 5-hydroxy-2-formylpyridine thiosemicarbazone. The synthesis was achieved by nitration of 1,4-dimethylisoquinoline (99) at the 5-position (100) followed by selective oxidation with selenium dioxide to the corresponding 1-carboxaldehyde (101). The aldehyde group was protected by conversion to the cyclic ethylene acetal (102), which was then catalytically



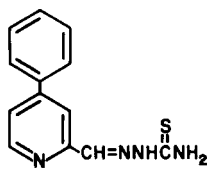
reduced to produce the 5-amino derivative (103). Reaction of (103) with thiosemicarbazide in the presence of hydrochloric acid led to the desired derivative (104). This was found to be an effective antineoplastic agent in mice bearing a

variety of transplanted tumours. In addition, evidence has been presented to support the view that compound (104), in contrast to 5-amino-1-formylisoquinoline thiosemicarbazone (63), is only minimally susceptible to enzymatic acetylation of the 5-NH_2 group. Such acetylation converts 5-amino-1-formylisoquinoline thiosemicarbazone to the 5-acetylamino derivative, an agent which has been shown to be completely devoid of antitumour activity [34]. Thus, the presence of a methyl group in the 4-position creates steric hindrance which prevents enzymatic conjugation of the 5-amino group. Compound (104) has been evaluated as an inhibitor of ribonucleoside diphosphate reductase and was found to be 60-fold more potent as an inhibitor of this enzyme from a rat hepatoma than was (17) [45] and twice as effective as 2-formyl-4-(3-aminophenyl)pyridine thiosemicarbazone, an agent reported earlier by this laboratory as a potential candidate for clinical trial [22]. Compound (104) appears to be superior in its properties to any other agent in this series of α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones reported to date and, therefore, possesses the requisite properties for consideration as a second generation drug of this class for trial against cancer of man.

X-RAY DIFFRACTION STUDIES

To complement synthetic approaches to studies of the relationship between structure and activity and to determine more precisely the molecular conformations and electronic distributions necessary for maximum inhibitory activity by these compounds, a series of single X-ray diffraction studies on α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones (both biologically active and inactive molecules) has been initiated [46]. 2-Formyl-4-phenylpyridine thiosemicarbazone (105), an agent possessing borderline biological activity [25], was selected initially to gain structural information to explain the lack of antineoplastic activity of this material; a reasonable control appeared to be the structurally similar agent, 2-formyl-4-(3-aminophenyl)pyridine thiosemicarbazone (106) which is one of the active agents of this class of compounds [22]. Furthermore, the presence of the amino function is not a requirement for activity in the isoquinoline series. Studies of the crystal structure of (105) revealed that the entire thiosemicarbazone molecule is planar [46] and must undergo two 180° rotations to change its conformation to one which allows it to act as a tridentate ligand. The effect of the insertion of the NH_2 group in the molecule has not yet been studied.

Mathew and Palenik [47] determined the crystal structure of bis(isoquinoline-1-carboxaldehyde thiosemicarbazanato)nickel(II) monohydrate; their studies indicated that distorted octahedral molecules are held together by a hydrogen-



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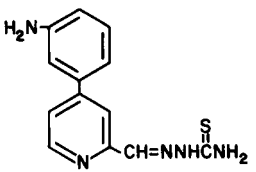

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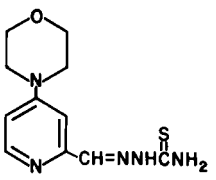


(107)

bond system involving N-H---N, N-H----O, and O-H----S hydrogen bonds. This study was followed by structural determinations of 4-formylpyridine thiosemicarbazones [48] and 2-formylthiophene thiosemicarbazone [49], 2 agents with inactivity against cancer. 5-Hydroxy-2-formylpyridine thiosemicarbazone (17) has been shown to be a planar molecule [50], in contrast to the structure of 4-formylpyridine thiosemicarbazone in which the side chain is twisted by 14° with respect to the plane of the pyridine ring [48]. It has been suggested [50] that the delocalization of the heterocyclic ring into the thiosemicarbazone side chain is responsible for the enhanced activity of (17) over aliphatic derivatives which are devoid of tumour-inhibitory activity. Such delocalization was suggested to influence the sulphur atom in a manner that would result in the formation of a specific set of ligands with preferential affinity for certain metal atoms, and therefore acts as a relatively specific agent for iron. However, since Brown and Agrawal [46] have shown the same degree of delocalization with the 4-phenyl analogue (105) that occurs with (17), the validity of this conclusion must be questioned.

Another study of an active antineoplastic agent, 2-formyl-4-morpholinopyridine thiosemicarbazone (107), revealed that the conformation of the molecule is similar to that observed with other related thiosemicarbazone derivatives [51]. Comparison of these molecular structures indicates that the rotational barrier about the 2-carbon of the pyridine ring and the aldehydic carbon, which is influenced by the dihedral angle of this bond, may be important for the antineoplastic activity of this class of agents.

METAL COMPLEXES

α-(*N*)-Heterocyclic carboxaldehyde thiosemicarbazones are excellent coordinating agents for a number of transition metals including divalent iron, cobalt, nickel, copper, zinc and manganese [9]. The exceptionally strong affinity of 2-formylpyrazine thiosemicarbazone for ferrous ions *in vivo* was demonstrated by the finding that administration of a 100 mg kg^{-1} dose of this compound to mice removed about 11 μg of iron per animal in 24 h [52]. Administration of 5-

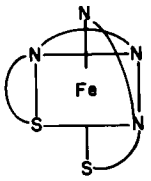
hydroxy-2-formylpyridine thiosemicarbazone (17) to patients with cancer leads to the excretion of significant amounts of iron (2 to 11 mg per patient per 24 h) in the urine, probably in chelate form with (17) [55,56]. Serum iron and total iron-binding capacity of the serum was increased in these individuals; however, urinary and serum calcium, copper and zinc levels remained within normal limits during treatment with this agent.

It has been hypothesized [57] that the biological activity of compounds of this class is associated with the chelation of iron and that isoquinoline (1) or pyridine (4) derivatives interact with ribonucleoside diphosphate reductase either by coordination of iron in the metal-bound enzyme or by the prior formation of the iron chelate of these agents which interacts with the target enzyme and is the active inhibitory form. Direct correlation between antitumour activity and the chelating ability of several of these compounds was shown by Michaud and Sartorelli [53]. The studies demonstrated that the capacity of 1-formylisoquinoline thiosemicarbazone to prolong the survival time of mice bearing Sarcoma 180 ascites cells and to inhibit the formation of DNA in these cells was decreased considerably by the substitution of an imino group for the sulphur atom in the thiosemicarbazone side chain, and it was eliminated completely by replacing the sulphur with an oxygen atom. These findings were correlated with the abilities of these derivatives to coordinate ferrous ions. 1-Formylisoquinoline thiosemicarbazone (1) had the highest chelation affinity, followed by the guanylhydrazone; the semicarbazone did not appear to bind ferrous ions. Similar results have been shown [54] in the pyridine series; in these investigations the 5-hydroxy analogue (17) was found to be a more active antineoplastic agent than its analogous selenosemicarbazone analogue, and the guanylhydrazone and semicarbazone derivatives of (17) were ineffective. The chelating potential of these agents correlated with biological activity: 5-hydroxy-2-formylpyridine thiosemicarbazone (17) was shown to be the most effective ligand in this series, the selenosemicarbazone was about 1/8th as effective, while the other 2 analogues, i.e., the guanylhydrazone and semicarbazone, were found to have very low chelating ability. Furthermore, these properties also corresponded with the degree of inhibition of DNA synthesis by these compounds in transplanted neoplastic cells.

It has been reported that the pyridine derivative (4) removes iron from ferritin to form the iron (III) complex [58]. This complex is rapidly reduced by haemoglobin and is only slowly reoxidized by oxygen in aqueous solution or plasma. Thus, both the iron (III) and iron (II) chelates seem to be stable in plasma, suggesting that the iron (II) complex may exist *in vivo*.

The synthesis of iron, cobalt and nickel complexes of 2-formylpyridine thiosemicarbazone has been reported [59]. The α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones strongly complex transition metals through their N*-N*-S*

tridentate ligand system (108, 109), iron forming an octahedral 1 : 2 metal to ligand complex whereas copper forms a 1 : 1 metal to ligand complex.



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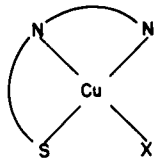

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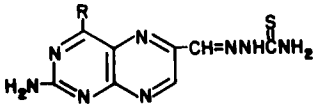


(110)

a. R = NH_2
b. R = OH

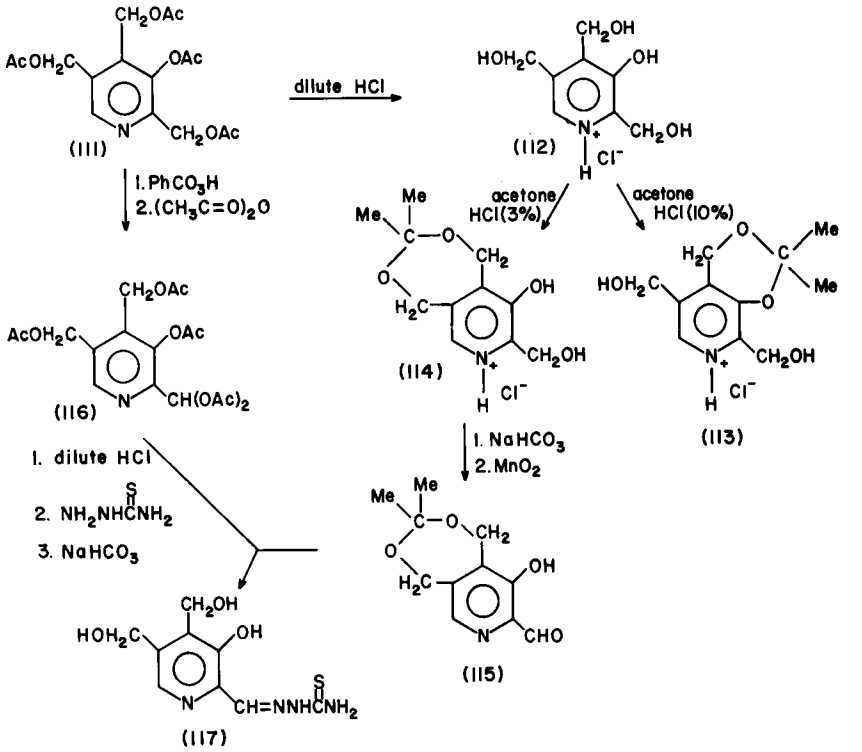
The possibility that certain metal complexes of α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones may be cytotoxic to tumour cells has been examined [58,60–62].

SITE-DIRECTED CHELATING AGENTS

Since it is conceivable that the chelating potential of the formyl thiosemicarbazone side chain, positioned alpha to a heteroaromatic ring nitrogen atom, might be directed toward a variety of metal-containing enzymes by modification of the heterocyclic ring systems such that it resembles the natural substrate of the target metalloenzyme, several appropriate modifications of the heteroaromatic nucleus have been carried out. The pteridine nucleus was one such ring system selected to direct the formyl thiosemicarbazone portion of the molecule to the enzyme dihydrofolate reductase [63]. Although dihydrofolate reductase has not been shown to be a metal-containing enzyme, it has been reported that the enzyme from neoplastic cells is inactivated by the metal-chelating agents *O*-phenanthroline and ethylenediaminetetraacetate [64]. 2,4-Disubstituted pteridine-6-carboxaldehydes were reacted with thiosemicarbazide to yield the desired thiosemicarbazones (110). Enzyme inhibition data indicated that the 2,4-diaminopteridine derivative (110a) was more potent than the 2-amino-4-hydroxypteridine compound (110b). However, when the sulphur atom in the thiosemicarbazone side chain was replaced by oxygen, the resulting derivative

was slightly better as an inhibitor of dihydrofolate reductase than the parent molecule [63]. These findings indicate that chelating potential was not associated with the enzyme inhibitory activity of these pteridines.

A second enzymatic site, which has been selected as a target to direct the chelating potential of the thiosemicarbazone molecule, is pyridoxal phosphokinase [65]. This catalyst is a zinc-requiring enzyme [66] that catalyzes the phosphorylation of pyridoxine at position 5 to form the active coenzyme form. In an effort to accomplish inhibition, 2-formyl-3-hydroxy-4,5-bis(hydroxymethyl)pyridine thiosemicarbazone (117) was synthesized [65]. This was accomplished by two different procedures. The common intermediate, 3-acetoxy-2,4,5-tris(acetoxymethyl)pyridine (111) [67], upon acid hydrolysis afforded 3-hydroxy-2,4,5-tris(hydroxymethyl)pyridine hydrochloride (112), which was treated with hydrogen chloride as a suspension in acetone [68]. The amount of hydrogen chloride taken up by the suspension is a critical factor and,



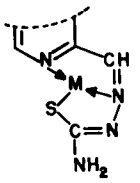
accordingly, yields are variable. When the quantity of hydrogen chloride utilized

was about 10%, only a six-membered ring ketal (113) was formed; however, 3% hydrogen chloride afforded the seven-membered ketal (114) and unreacted compound (112). Compound (114) as the free base was then oxidized with manganese dioxide to form the corresponding aldehyde (115). Compound (115), after hydrolysis with dilute hydrochloric acid and subsequent reaction with thiosemicarbazide and neutralization with sodium bicarbonate, produced the desired compound (117). An alternative route produced a better yield of (117). This procedure required the initial formation of the N-oxide of (111) which, upon rearrangement with acetic anhydride, yielded the pentaacetate (116) [67]. After acid hydrolysis of the pentaacetate, the solution was reacted with thiosemicarbazide, to form the desired compound (117) upon neutralization with sodium bicarbonate.

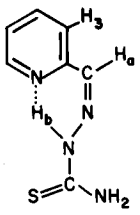
Evaluation of the antineoplastic activity of (117) against Sarcoma 180 and L-1210 leukemia in mice resulted in the demonstration of significant activity. The presence or absence of pyridoxine hydrochloride in the diet did not influence the degree of inhibition of the growth of Sarcoma 180 ascites cells. In addition, compound (117) was inactive against a subline of Sarcoma 180 resistant to 1-formylisoquinoline thiosemicarbazone (1), suggesting that these agents may have a similar biochemical mode of action. In keeping with this expectation, compound (117) inhibited the synthesis of DNA, but not of RNA or protein in Sarcoma 180 ascites cells *in vitro*.

GEOMETRIC ISOMERS

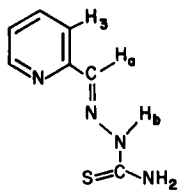
The geometrical isomers of the thiosemicarbazones of 2-formylpyridine (4) and 1-formylisoquinoline (1) have been synthesized and their structures studied by spectroscopic methods [69]. X-Ray diffraction studies of bis(isoquinoline-1-carboxaldehyde thiosemicarbazanato)nickel(II) monohydrate by Mathew and Palenik [49] have demonstrated that the transition metal binds with the thiol form (118) of the α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones. The



(118)



(119) Z



(120) E

original postulation of French and Blanz [9] envisioned that the thiosemicarbazone would be in the Z (syn) form for chelation; however, Mathew and Palenik [49] established that in the chelate form the aldimine bond is in the E (anti) configuration. Separation and isolation of these 2 isomers was accomplished by thin-layer chromatography (silica gel) [69]. The faster moving isomer was assigned the Z (syn) configuration (119) and the slower moving isomer the E (anti) configuration (120). A very large downfield shift of the imino proton H_b (δ 14.15) was found in the Z isomer which was attributed to intramolecular hydrogen bonding. In addition, the ring protons of (119) (H_4, H_5, and H_6) are deshielded relative to the same protons of (120). This finding is consistent with a withdrawal of electron density from the pyridine ring via the N atom involved in hydrogen bonding. The presence of intramolecular hydrogen bonding in (119) was also confirmed by UV spectra; Z isomers have an absorption maximum at longer wavelengths (332 nm) than the E isomers (323 nm). This study also concluded that the E isomer predominates in the synthetic preparation and that in solution and in the presence of some metal ions the E isomers probably exist in equilibrium with the tautomeric S-H form. Furthermore, the isomers are labile, in that they are converted to a mixture of the geometrical isomers within a relatively short time, whereas in the dry state they are stable for several days.

BIOCHEMICAL MECHANISM OF ACTION

The α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones are primarily inhibitors of the synthesis of DNA in neoplastic cells [57,70]; therefore, they exert their effect in the S phase of the cell cycle [71,72]. Inhibition of the biosynthesis of RNA and protein is also produced by agents of this class; however, these metabolic processes are considerably less sensitive than is the replication of DNA [57,70,73–75]. Interference with the biosynthesis of DNA by these agents was shown to be due to inhibition of the enzyme ribonucleoside diphosphate reductase [70,73,74,76]. The α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones constitute, as a class, the most potent known inhibitors of ribonucleoside diphosphate reductase, being 80–5000 times more effective than the classical inhibitor of this enzyme, hydroxyurea [for appropriate references see 77].

The kinetic mechanism of inhibition of ribonucleoside diphosphate reductase by α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones is not clear. The concentrations of the nucleoside diphosphate substrate, the allosteric activator ATP, or magnesium ion do not influence the inhibition of the enzyme produced by all of the thiosemicarbazones tested to date. Interesting differences exist, however, between the ring hydroxylated and nonhydroxylated α-(*N*)-heterocyclic carbox-

aldehyde thiosemicarbazones with respect to the dithiols, used as model substrates in place of the natural substrate thioredoxin, and to iron. 1-Formylisoquinoline thiosemicarbazone (1) and 2-formylpyridine thiosemicarbazone (4) are similar in action; both are partially competitive with the dithiol substrate and both prevent any stimulation of reductase activity by iron. It has been hypothesized [73,74] that inhibition by these derivatives is the result of either coordination of iron in the metal-charged enzyme by the inhibitor, or formation of an iron chelate of (1), which acts as the true inhibitor at or near the site functionally occupied by thioredoxin.

Studies conducted on the inhibition of ribonucleoside diphosphate reductase by the preformed iron chelate of 1-formylisoquinoline thiosemicarbazone (1) [(Fe)IQ-1] have shown that under appropriate conditions (Fe)IQ-1 was essentially equal to (1) as an inhibitor of enzymatic activity [78]. At a concentration of (Fe)IQ-1 which inhibited enzymatic activity by 73% in the absence of added Fe^{2+}, only 30% inhibition was observed when Fe^{2+} was added to the preparation. In contrast, (1) decreased enzyme activity 65% in the presence of Fe^{2+} but only 15% without Fe^{2+}. The findings imply that Fe(IQ-1) is the active form of the inhibitor and stress further complexities in the mode of inhibition.

The hydroxylated derivatives, 5-hydroxy-2-formylpyridine thiosemicarbazone and 3-hydroxy-2-formylpyridine thiosemicarbazone, show a different pattern of inhibition [74]. They appear 'competitive' with iron and either non-competitive or uncompetitive with the dithiol substrate; the imprecise nature of the assay does not allow a choice between these alternatives. The failure of the dithiol to reverse inhibition of ribonucleoside diphosphate reductase by the hydroxylated derivatives implies that the interaction of these inhibitors with the enzyme occurs at a site different from that involved in the action of (4) and (1). The impure nature of this complex enzyme system, however, makes it impossible to explain fully these differences and further advances will require the availability of a highly purified enzyme.

Some of the structural features required for inhibition of ribonucleoside diphosphate reductase have been determined [30]. These studies suggested that position 6 of (4) and position 3 of compound (1) are equivalent with respect to orientation of the inhibitor at the enzymatic binding site and that little or no tolerance exists for modification at this position. In addition, substitution of the terminal amino group of the thiosemicarbazone side chain decreased enzyme inhibition, suggesting the presence of a low bulk tolerance zone in this position [23]. The results also indicated that the isoquinoline derivative (1), which can be visualized as a pyridine derivative with a benzene ring fused across the 3- and 4-positions, is about 6-fold more potent as an inhibitor of the enzyme than is (4). Likewise, introduction of a Me group on the pyridine ring of (4) at either the 3-, 4-, or 5-positions resulted in derivatives that were better inhibitors of ribo-

nucleoside diphosphate reductase activity than the pyridine derivative (4). These findings suggest the possible existence of a hydrophobic bonding zone adjacent to the inhibitor-binding site of the enzyme.

Although the primary biochemical lesion induced by the α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones appears to be at the level of the enzyme ribonucleoside diphosphate reductase, and studies of structure-activity relationships have indicated the requirement for inhibition of this enzyme for antineoplastic activity [1], recent studies from our laboratory have shown that (1) interacts directly with Sarcoma 180 DNA and causes single strand breaks in DNA [79]. This agrees with the findings of Karon and Benedict [71] who have reported that 5-hydroxy-2-formylpyridine thiosemicarbazone caused chromatid breaks, primarily during the S-phase, in hamster fibroblasts. The precise mechanism(s) responsible for the fragmentation of DNA is unknown; however, it would not appear to be due completely to inhibition of the activity of ribonucleoside diphosphate reductase [57]. Regardless of the precise mechanism by which these agents induce breakage of tumour cell DNA, this second site would appear to be of major significance to the cytotoxic action of α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones, since it creates a lesion in the genome that is reinforced by blockade of ribonucleoside diphosphate reductase.

DISTRIBUTION AND METABOLISM

The metabolism and distribution of α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones have been investigated in mice, dogs, and man. The tissue distribution of radioactive 1-formylisoquinoline thiosemicarbazone (1) has been studied in mice [35,36], and the greatest amount of radioactivity was found in the intestine (24 to 40%), liver (4 to 12%), and stomach (2 to 6%). Lesser amounts were present in other tissues. The relatively large amounts of radioactivity from (1) present in the tissues of the gastrointestinal tract of the mouse have also been reported for 5-hydroxy-2-formylpyridine thiosemicarbazone in the dog [80]. These findings suggest that the localization of these agents in the gastrointestinal tract may be in part associated with the nausea, vomiting and diarrhoea that characterize the toxicity of 5-hydroxy-2-formylpyridine thiosemicarbazone in man [55,56].

Twenty percent of the radioactivity from (1) labelled in the side-chain was excreted in the urine of mice and 2.8% in the faeces in 16 h [35]. During an 8-h period, about 2% of the radioactivity was present in the respiratory CO_2 when side-chain labelled (3′-^{14}C), but not ring labelled (1-^{14}C) 1-formylisoquinoline thiosemicarbazone, was administered. Furthermore, these workers found that the half-life of radioactivity from (1) in the blood of dogs was about 4 h, and

between 28 and 46% of the label was excreted in the urine during a 48-h period.

Investigation of urinary metabolites indicated great complexity in the degradative pattern of 1-formylisoquinoline thiosemicarbazone (1). Little unchanged drug was present in urine, indicating the completeness of breakdown. About 60% of the excreted derivatives of (1) underwent desulphuration, a change that should lead to loss of antitumour potency, since 1-formylisoquinoline semicarbazone has been shown to be inactive against transplanted murine neoplasms [15]. Metabolic cleavage of the side chain of the isoquinoline derivatives (1) is of lesser importance accounting for 11 to 16% of the total urinary metabolites in the dog and about 15% in the mouse [35]. Two types of cleavage occur, one on the =N-N- linkage and the other on the -CH=N- bond. Thus, about 4% of the urinary radioactivity was in the form of urea, and somewhat less as thiourea, indicating that desulphuration need not precede side-chain cleavage. Significant amounts of semicarbazide and thiosemicarbazide were also found.

The water solubility of urinary metabolites of (1), which contrasts with that of the parent compound, suggests that modification of the ring occurred. This was supported by the finding of (a) a hydroxyl infrared band in an acidic metabolite and (b) 13.3% of urinary radioactivity in glucuronide form.

5-Amino substituted isoquinoline derivatives have been shown to be susceptible to acetylation [45], a process expected to result in inactivation of tumour-inhibitory derivatives.

In the dog, radioactivity from 5-hydroxy-2-formylpyridine thiosemicarbazone appeared in both the bile and urine, where 2 unidentified metabolites were separated [80]. In man, plasma levels of this 5-hydroxylated pyridine derivative decayed in a biphasic mode with an initial half-life of 2.5 to 10 min [55]. This is shorter than the $t_{1/2}$ of 15 min for this drug in mice [81]. Forty-seven to 75% of a therapeutic dose of 5-hydroxy-2-formylpyridine thiosemicarbazone was excreted by patients with cancer; 50 to 74% of the urinary radioactivity was in the form of glucuronide conjugates and the remaining material was that of an unknown compound [55]. No degradation of the thiosemicarbazide side chain of this drug appeared to occur in man, which contrasts to that found with compound (1) in the dog. This finding did not appear to be due to a species difference, but a reflection of differing susceptibilities of various α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones to metabolic alteration.

PHASE I CLINICAL STUDIES

5-Hydroxy-2-formylpyridine thiosemicarbazone is the only member of this series that has been administered to man as part of a Phase I study [55,56]. The selec-

tion of this agent for clinical trials was due to (a) its considerable activity against transplanted tumours and a spontaneous dog lymphoma, and (b) its ease of parenteral administration as its sodium salt. The results of the two independent Phase I studies showed that transient decreases in blast counts occurred in 6 of 25 patients with leukemia, while no antitumour effects were observed in 18 patients with solid tumours. Administration of relatively large doses of drug was limited primarily by gastrointestinal toxicity, although myelosuppression, haemolysis, anaemia, hypertension, and hypotension also occurred in patients treated with the most aggressive regimens. The clinical impression was that the gastrointestinal toxicity was centrally mediated; however, there is no direct evidence to support this supposition. Thus, the impressive antineoplastic activity of the 5-hydroxypyridine derivative in animal systems was not achieved in man, although it must be stressed that in this instance, as in all Phase I investigations, far advanced patients were employed. The relative inactivity of 5-hydroxy-2-formylpyridine thiosemicarbazone as an inhibitor of tumour growth in man appeared to be the result of (a) its relatively low inhibitory potency for the target enzyme, ribonucleoside diphosphate reductase, compared to the most potent member of this class, compound (1), being about 100-fold less effective, and (b) its short biological half-life in man because of the rapid formation and elimination of the *O*-glucuronide conjugate.

CONCLUSIONS

α-(*N*)-Heterocyclic carboxaldehyde thiosemicarbazones have been shown to be potent inhibitors of the biosynthesis of DNA in mammalian cells. Studies with transplanted murine neoplasms have demonstrated that the enzymatic site responsible for the blockade of DNA replication is at the level of ribonucleoside diphosphate reductase, an enzyme of critical importance for the generation of the deoxynucleoside triphosphate precursors of these macromolecules. Kinetic studies have been carried out with a partially purified enzyme from a rat tumour in an effort to define the molecular mechanism by which agents of this class inhibit the activity of ribonucleoside diphosphate reductase. The concentrations of the nucleoside diphosphate substrate, the allosteric activator ATP, and magnesium ion did not influence inhibition by a representative isoquinoline derivative (1) of this class. Dithiothreitol, a model dithiol used in place of the natural substrate thioredoxin, however, was partially competitive with the inhibitor. The results appear to be most compatible with a model in which the preformed iron chelate of (1) interacts with the enzyme at or near the site occupied by the dithiol substrate. To ascertain some of the structural features required for inhibition of ribonucleoside diphosphate reductase activity, the

inhibitory potency of a series of methyl and benzo derivatives of (4) has been ascertained. The results suggested that position 6 of (4) and position 3 of (1) are equivalent with respect to orientation of the inhibitor at the enzymatic binding site and that little or no tolerance exists for structural modification at this position. In a similar manner, substitution of the terminal amino group of the thiosemicarbazone side chain decreased enzyme inhibition, supporting the presence of a low bulk tolerance zone in this position. Further studies with these substituted derivatives were interpreted to be suggestive of the existence of a hydrophobic bonding zone adjacent to the inhibitor-binding site of the enzyme. These results have been employed to design 4-methyl-5-amino-1-formylisoquinoline thiosemicarbazone [45], a second generation derivative of this class with strong inhibitory potency for ribonucleoside diphosphate reductase, as well as the other necessary requisite properties for clinical trial as an antigenoplastic agent.

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8 Prostaglandins and Thromboxanes

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INTRODUCTION

Since the previous review on this topic [1], continued widespread interest in the prostaglandins has led to many advances in the chemistry, biology and clinical evaluation of these substances and their first commercial application has been established with the marketing of prostaglandins E_2 and $F_{2\alpha}$ for the induction of labour in women.

There is now a better understanding of the physiological role of prostaglandins and of major significance has been the discovery [2] that aspirin, indomethacin and other non-steroid anti-inflammatory agents probably exert their therapeutic effects by inhibiting prostaglandin biosynthesis. Several new naturally occurring prostaglandins have been identified and particular interest has centred around

recently discovered thromboxanes [3] and prostacyclins [4] which have a key role in the regulation of platelet aggregation.

Many prostaglandin analogues have been synthesized and subjected to biological testing, and clinical studies have been extended from the earlier work on the natural compounds to the evaluation of modified prostaglandins with greater potency and selectivity of action.

An attempt is made here to update the earlier article [1] by reviewing major advances in prostaglandin chemistry and biological activity and to indicate the extent to which research has progressed towards the realisation of earlier hopes of the discovery of new therapeutic agents. The review has been extended to include the thromboxanes which, although as strictly defined, are not prostaglandins, are closely related to them in origin, chemistry and biological properties.

Several books and reviews [5–11] and publications based on symposia [12–16] afford more detail on many aspects of the subject which are excluded or receive only brief reference here. Much of the original work on prostaglandins is now reported in the monthly journal 'Prostaglandins' inaugurated in 1972 and published by Geron-X, Los Angeles, California, which affords a medium for the rapid publication of all aspects of research in this area.

CLASSIFICATION AND NOMENCLATURE

Prostaglandins have generally been regarded as derivatives of prostanoic acid (*Figure 8.1*) and classified according to the number, type and arrangement of oxygen functions and double bonds which are built into this basic system. The designation of further naturally occurring prostaglandins has extended the number of groups from the four, PGE, Fα, A and B, described in the previous review [1] to a total of nine, PGA to I, together with the related thromboxanes of which there are two types, TXA and B.

Of the newly designated types, the PGCs were discovered as an intermediate stage in the biosynthetic conversion of the A to the B series [17], the PGDs [18] are the compounds referred to previously as 11-dehydro compounds [1, p. 326], and the G and H prostaglandins are the endoperoxides, originally postulated as intermediates in the biosynthesis of the primary prostaglandins (PGs E and F) and included in the prostaglandin classification following their isolation and identification as distinct chemical entities [19]. PGI is the name now officially given to prostacyclin [20], which, when first discovered, was designated as PGX. Thromboxanes are distinguished from the prostaglandins by the presence of a tetrahydropyran in place of the cyclopentane ring [3]. Both PGI and the thromboxanes are formed from PGs G and H as alternative pathways to the primary prostaglandins.

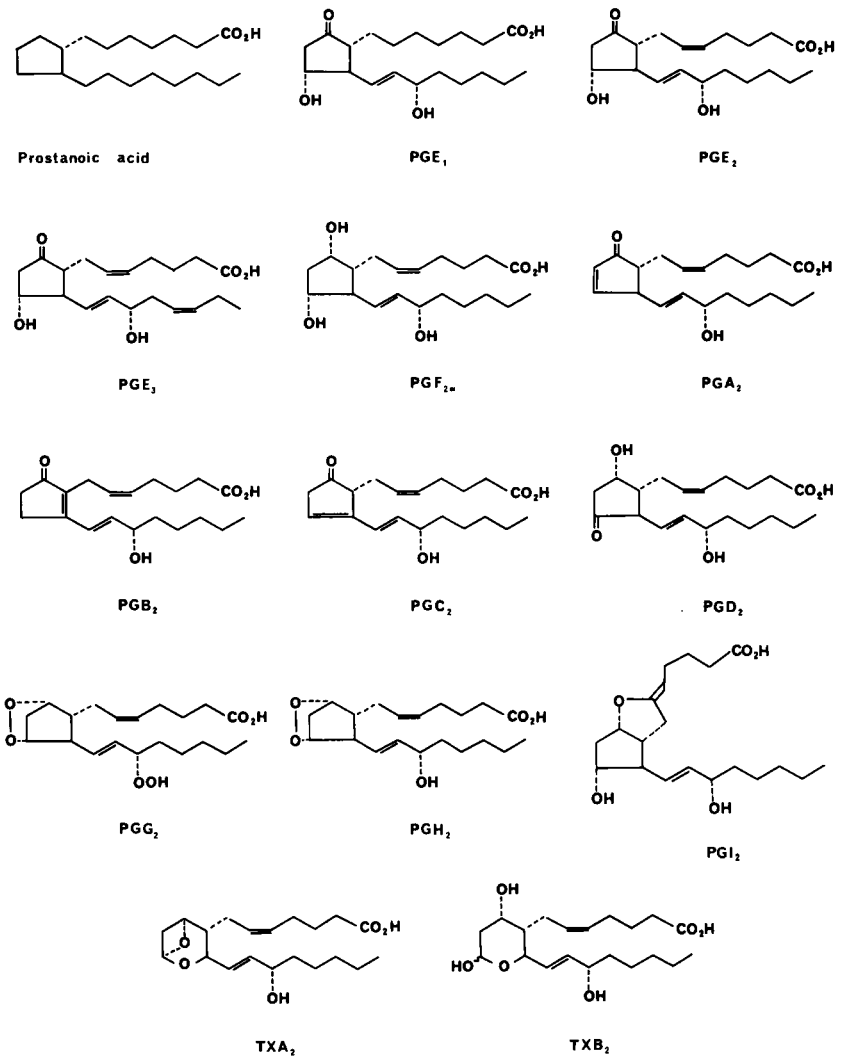


Figure 8.1. Prostaglandins and thromboxanes: structure and nomenclature

Individual members of each group are characterised by the number of double bonds in the side chain which are denoted by subscript numerals 1, 2 or 3. The three double bond arrangements, which are the same in all of the classes *, are

* Although in some cases all the possible compounds have not been isolated.

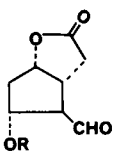
shown in *Figure 8.1* for the E series, the other groups being illustrated by the '2' compounds only. In the case of the F series a further subscript, α, is added to define the stereochemistry of the C-9 hydroxyl group.

CHEMICAL SYNTHESIS

The literature on the synthesis of prostaglandins is now very substantial, the number of published papers having increased some eight-fold since the last review [1]. Major advances have included new and improved syntheses of the primary (E and F) prostaglandins, syntheses of PGs A, C and D, thromboxane B_2 and prostacyclin (PGI), and the preparation of numerous prostaglandin analogues. Selected references are included here with the object of illustrating the main trends in this work.

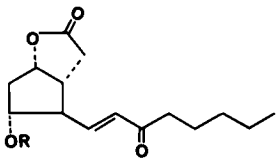
PROSTAGLANDINS E AND F

(a). Methods based on the Corey lactone intermediates. The synthetic approach by Corey based upon the lactone aldehyde (1a) and its elaboration *via*



(1)

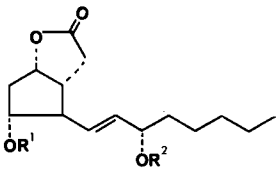
a, R= Ac
b, R= COC_6H_4Ph
c, R=Thp
d, R=COPh



(2)

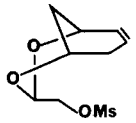
a, R=OAc
b, R=Thp
c, R=H

a, $R^1 = R^2 = Thp$
b, $R^1 = R^2 = H$
c, $R^1 = H$, $R^2 = Bu^t$



(3)

(4)



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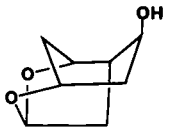
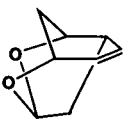


(6)

a Wittig reaction to enone (2a), conversion to (3a, Thp = tetrahydropyranyl) and then reduction to the lactol followed by a second Wittig reaction has dominated routes to the primary prostaglandins. The approach affords versatile routes to all members of the E and F series and has been widely used for the synthesis of these prostaglandins and their analogues. Many of the new prostaglandin syntheses involve novel approaches to intermediates of this type which are then carried forward essentially as before [1, p. 329].

Ernest, Woodward and colleagues employed the related lactol intermediate (10a) [21]. This was synthesized from the tricyclic lactone (4) prepared from *cis*-1,3,5-cyclohexanetriol and glyoxylic acid which was converted in three steps – borohydride reduction, mesylation and treatment with potassium hydroxide – to the bicyclic olefin mesylate (5). Solvolysis of the latter to give (6) and mesylation followed by reaction with potassium hydroxide then afforded the tricyclic olefin (7) which contains in a mutually and internally protected form the hydroxy group and the five-membered ring acetal of (10a) as well as a double bond in a suitable position for ring contraction and formation of the aldehyde function. Conversion to (10a) was then achieved by epoxidation using the reagent prepared from hydrogen peroxide and benzonitrile in methanol which gave the desired isomer as the major product, and ammonolysis to give the aminoalcohol (8), reacetalisation by standing with methanolic hydrogen chloride affording (9) and then diazotisation followed by spontaneous transformation of the diazonium compound. The synthesis of resolved (10a) and hence the natural prostaglandins was achieved by resolution at the alcohol (6) stage by formation of the *S*-ketopinic ester.



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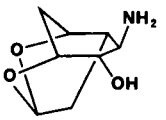

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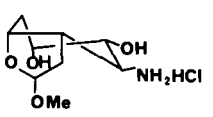

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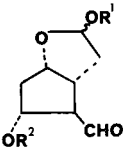
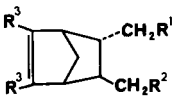


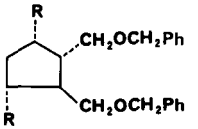
(10)

a, R^1 = Me, R^2 = H
b, $R^1 = R^2$ = H
c, R^1 = Me, R^2 = $CONHC_6H_4Ph$-p
d, R^1 = Cyclohexyl, R^2 = H



(11)

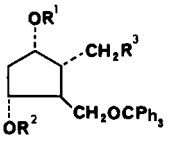
a, $R^1 = R^2 = OCH_2Ph$, R^3 = H
b, R^1 = CN, R^2 = OMe, R^3 = H
c, R^1 = CN, $R^2 = OCH_2Ph$, R^3 = Me



(12)

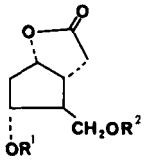
a, R = CHO
b, R = OAc

A British team [22] subjected the norbornene system (11a) to cleavage by reductive ozonolysis, affording the dialdehyde (12a) which has all the correct stereochemical features required in the prostaglandin. The dialdehyde (12a) was then converted by the sequence – reaction with methyl lithium, Jones oxidation and Baeyer–Villiger oxidation – to the diacetoxy compound (12b) and the latter, after debenzylation, subjected to an acetate rearrangement by refluxing in



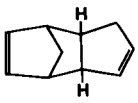
(13)

a, $R^1 = R^2$ = Ac, $R^3 = OCPh_3$
b, R^1 = H, R^2 = Ac, R^3 = OAc
c, $R^1 = R^2$ = Ac, R^3 = CN



(14)

a, $R^1 = R^2$ = H
b, R^1 = Ac, R^2 = Me
c, R^1 = Thp, R^2 = Me
d, $R^1 = COC_6H_4$-p-Ph, R^2 = H
e, R^1 = Ac, R^2 = H



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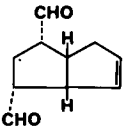
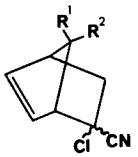


(16)

pyridine to give an equilibrium mixture of two acetates which was converted to their trityl derivatives (13a) and (13b). The primary acetoxy compound (13b) was then taken forward (by deacetylation, selective tosylation of the primary alcohol, reacetylation and reaction with sodium cyanide) to the nitrile (13c) which afforded the lactone diol (14a) after removal of the protecting groups and nitrile hydrolysis. Lactone (14a) and the methoxy lactone (14b), which was synthesized by a variation of this procedure, have been previously taken forward to prostaglandins by Corey [1, refs. 461, 116].

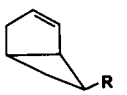
Katsube and Matsui [23] achieved a synthesies of (1a) by a related sequence starting from (11b) and Hoffman–La Roche chemists [24] have described a route based on (11c). In an approach by a Reckitt and Colman team [25], the endo-dicyclopentadiene (15) was cleaved to the dialdehyde (16) and thence transformed *via* a second cleavage and epimerisation to afford the aldehyde (10b).

In another approach by ICI chemists [26–28], the aldehyde (1b) has been synthesized by a modification of the original route used by Corey *via* the bicycloheptene (17a) which was constructed by Diels–Alder addition of 2-chloroacetonitrile and 5-methoxymethyl-1,3-cyclopentadiene [1, p. 329]. In the ICI method the latter was replaced by 6-acetoxyfulvene to give the adduct (17b) which was then hydrolysed with hydrochloric acid in acetone to the *anti*-aldehydes (17c) and the latter isomerised to the *syn*-aldehydes (17d) by



(17)

a. R^1, R^2 = H, CH_2OMe
b. R^1, R^2 = CHOAc
c. R^1 = H, R^2 = CHO
d. R^1 = CHO, R^2 = H

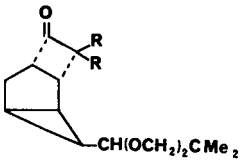


(18)

a. R = CHO
b. $R = CH(OCH_2)_2CMe_2$

prolonged heating with hydrochloric acid in dioxan. The dimethyl acetal of (17d) was then taken forward to the dimethyl acetal of (1b) *via* the procedures previously used by Corey [1, p. 329] and the acetal hydrolysed to (1b) using a two phase system of hydrochloric acid and 2% propan-2-ol in chloroform. An advantage of this method is that isomerisation of the 5-substituted cyclpenta-1,3-diene, which is a problem in the formation of (17a), is prevented here by the presence of the exocyclic enol acetate group in the acetoxyfulvene. This method also has the asset that the enol acetate grouping which becomes a formyl group in aldehyde (1b) is introduced at the correct oxidation level. In a further development of this work [28] it was shown that by carrying out the Wittig reaction to form the prostaglandin ω-side chain at the bicycloheptene aldehyde (17d) stage, the latter can be converted directly into (±)-$PGF_{2\alpha}$ in seven steps without the use of protecting groups.

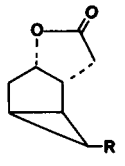
The original Upjohn approach to primary prostaglandins based upon the cleavage of bicyclo[3,1,0]hexane intermediates [1, p. 332] has now been applied to the synthesis of the lactone (3b) [29–33]. For this purpose the



(19)

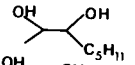
a, R = Cl

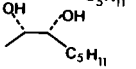
b, R = H

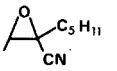


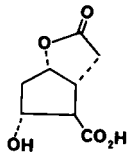
(20)

a, R=CHO

b, R = 

c, R = 

d, R = 



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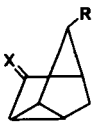
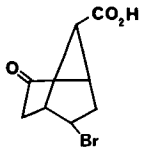


(22)

a, X = H, OCHO, R=CH_2OCHO

b, X = O, R=CO_2H



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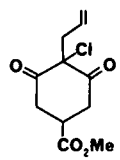


(24)

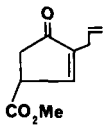
bicyclo[3,1,0]hexanediols (20b,c) were synthesized from the aldehyde (18a), available from oxidation of norbornadiene, which was converted to the acetal (18b) and treated with dichloroketene to give the dichlorocyclobutanone (19a). Dehalogenation with zinc and ammonium chloride to give (19b), Baeyer–Villiger oxidation and hydrolysis of the acetal with formic acid then led to the aldehyde (20a) which afforded a mixture of the diols (20b) and (20c) on Wittig elaboration and *cis*-dihydroxylation, performed conveniently using a tertiary amine oxide with osmium tetroxide as catalyst [29,30]. The diols underwent cleavage to lactone (3b) and the corresponding C-15 * epimer with dry formic acid and then sodium bicarbonate in methanol [31]. It was later shown [31] that the orthopropionate of (20b) undergoes cleavage with complete retention of the C-15 * stereochemistry affording a stereoselective route to (3b). The intermediates may be resolved at the aldehyde (18a) or ketone (19b) stages with l-ephedrine, thus affording the prostaglandins of natural configurations [29,32]. The aldehyde (20a) has also been taken forward by reaction with α,α-dibromoheptanenitrile and hexamethylphosphorus triamide, solvolysis of the product (20d) in formic acid and formate hydrolysis in sulphuric acid to give the cyanohydrin of (2c) which gave (2c) on hydrolysis with sodium bicarbonate [33].

Peel and Sutherland [34] synthesized the lactone acid (21) starting with the formation of the epimeric diformates by a Prins reaction of norbornadiene and paraformaldehyde to give (22a). Hydrolysis and Jones oxidation of (22a) then gave the keto acid (22b) which underwent ring opening with hydrogen bromide in acetic acid to the bromo compound (23). Baeyer–Villiger oxidation of the latter and addition of sulphuric acid to the reaction mixture followed by work up with alkali afforded an overall conversion to (21) of which the *p*-bromophenacyl ester was converted by the sequence – *p*-phenylbenzoylation, reduction with zinc and acetic acid and diborane reduction – to the alcohol (14d), previously taken forward to the aldehyde (1b) by Corey.

In a route by Hoffman–La Roche workers [35] ring contraction of the cyclohexane dione (24) with sodium carbonate in mesitylene gave the cyclopentenone (25) which was elaborated to the lactone (26a) by 1,4 addition of nitromethane, oxidative cleavage of the allyl side chain, ketone reduction and lactonisation.

* Prostaglandin numbering.

Conversion of the nitromethyl to aldehyde by oxidation of the lithium nitronate in aqueous sodium tetraborate with sodium permanganate followed by the usual



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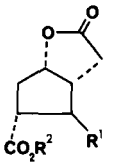
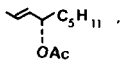
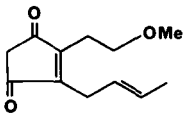


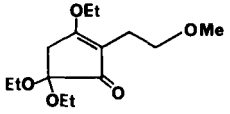
(26)

a. $R^1 = CH_2NO_2$, $R^2 = Me$

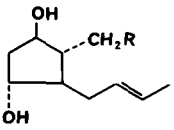
b. R^1 =  , $R^2 = OCOC_6H_4Cl\text{-}\underline{m}$



(27)



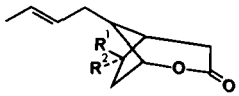
(28)



(29)

a. $R = CH_2OMe$

b. $R = CO_2H$



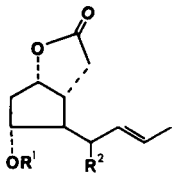
(30)

a. $R^1 = OH$, $R^2 = H$

b. $R^1 = H$, $R^2 = OAc$

Wittig elaboration and ketone reduction, ester hydrolysis, acetylation and treatment with dicyclohexylcarbodiimide and *m*-chloroperbenzoic acid afforded the mixed peranhydride (26b). Transformation of (26b) to (3b) was achieved by means of a carboxy inversion reaction which occurred, with retention of configuration, on refluxing in acetonitrile, and treatment of the resulting mixed carbonate with lithium methoxide.

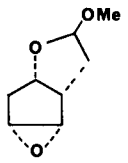
Workers at the University of Ghent [36,39] have described an approach involving the reduction of 2,3-dialkylcyclopentenones. Thus the cyclopentenedione (27) prepared by an organozinc reaction with crotyl bromide on (28) followed by acid hydrolysis, was reduced with zinc in acetic acid to give a mixture of cyclopentenolones which was reduced further with lithium in liquid ammonia–tetrahydrofuran in the presence of phenol to give a mixture of cyclopentane diols containing 60 per cent of the desired all-*trans* isomer (29a). The diacetate of (29a) on ether cleavage, Jones oxidation and removal of acetate functions afforded the carboxylic acid (29b), the lactone (30a) of which was inverted at the free hydroxyl by reaction of the tosylate either with hydroxylamine to give lactone (31a), or with tetraethylammonium acetate in acetone to give the acetate (30b) which afforded (31a) on acid hydrolysis. Lactone (1c) was then obtained on allylic bromination of the tetrahydropyranyl ether of (31a), hydrolysis with silver carbonate and sodium periodate–osmium tetroxide cleavage. Another useful approach has arisen from the finding that the prostaglandin ω-chain may be attached to the 5-membered ring by means of a regioselective cleavage of a suitably substituted cyclopentane epoxide. Thus Corey, Nicolaou and Beames [40] achieved the opening of the epoxidolactol methyl ether (32) with divinylcopper–lithium to give (33) as the major product of which the *p*-phenylphenyl urethane derivative afforded the lactol methyl ether (10c, exo-isomer) by an olefinic cleavage with sodium metaperiodate–osmium



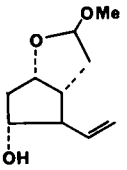
(31)

a, $R^1 = R^2 = H$

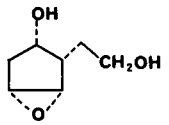
b, R^1 = Thp, R^2 = Br



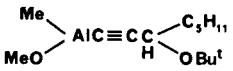
(32)



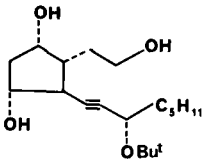
(33)



(34)



(35)



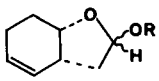
(36)

tetroxide. Fried and Sih [41,42] found that the diol epoxide (34) underwent alkynylation with the alanane (35) to give the triol (36), which could be taken through to lactone (3c) by debutylation, reduction of the triple bond with lithium aluminium hydride and then a selective dehydrogenation with oxygen and platinic oxide.

Yet another approach by Corey and Snider [43] utilised a novel allylic functionalisation of the acetal (37) with *N*-phenyltriazolinedione to give the intermediate (38), the *N*-methyl derivative of which afforded the hydrazine (39a) on dihydroxylation of the olefinic bond and hydrolysis of the urazole moiety with potassium hydroxide. Hydrogenolysis then led to the amine (39b) which underwent ring contraction to (10d) on treatment with sodium nitrite in aqueous acetic acid.

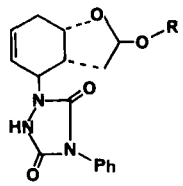
Corey and Ensley [44] have also introduced a modification of an earlier approach to lactones (1) from bicycloheptane intermediates [1, p. 329] which involves an asymmetric induction and thus obviates the need for a resolution stage.

A route to the optically active lactone alcohol (14e) has been reported by Dow and Gruppo Lepetit chemists [45] in which the intermediate (40) was constructed from *(S)*-(−)-malic acid and cyclised in a buffer of triethanolamine–triethanolamine hydrochloride to give the cyclopentenone (41) as the major product. The latter was hydrogenated to the cyclopentanone (42) which was then taken foward by ketone reduction to the α-hydroxyl, hydrolysis and



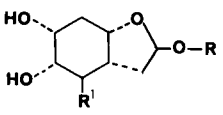
(37)

R = cyclohexyl

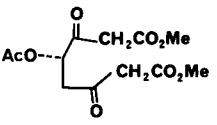


(38)

R = cyclohexyl



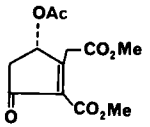
(39)



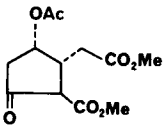
(40)

a. R = cyclohexyl , R^1 = NHNHMe

b. R = cyclohexyl , R^1 = NH_2



(41)

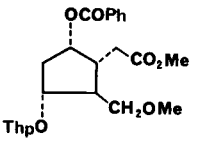


(42)

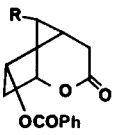
cyclisation to give (21) which afforded (14e) on conversion to the acetate and then reduction of the acid chloride with sodium borohydride.

Some useful improvements have been reported in the stages by which the lactone intermediates are converted to the prostaglandins. Corey, Becker and Varma [46] have developed a stereoselective reduction of the unsaturated ketones to the desired 15(*S*) alcohols of type (3) which hitherto were obtained together with an approximately equal amount of the 15(*R*) isomer. Stereochemical control was achieved by carrying out the reduction with a bulky borohydride reagent and by attaching a linear directing group to the C-11 ring oxygen. Reagents of this type have the additional advantage that they minimise conjugate reduction of the double bond, for which purpose aluminium isopropoxide is also effective [47].

Ono chemists [48] have devised a new route to $PGF_{2\alpha}$ from the precursor (14b) of the lactone aldehyde (1a) which avoids the difficulty of operating with the latter due to its thermolability. In the Ono procedure (14b) was converted *via* acetate hydrolysis to (14c) which was subjected to lactone cleavage and the resulting hydroxy acid converted to the ester (43). The latter was then saponified, the tetrahydropyranyl group removed and the product relactonised to (44a) which was then taken forward to $PGF_{2\alpha}$ *via* the lactone aldehyde (44b) and Wittig elaboration, essentially by the procedure used by Corey for elaboration of (1a). The lactone aldehyde intermediate (44b) used in this route was found to be much more stable than (1a) and easy to handle.



(43)



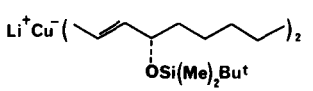
(44)

a. R = CH_2OMe

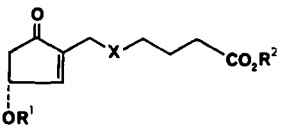
b. R = CHO

In further variations of this general approach, the synthesis has been modified so that the carboxylic side chain is added before the ω-chain [27,49].

(b). Methods based upon conjugate addition of organometallic complexes to cyclopentenones. Outstanding among newer approaches to the natural prostaglandins are methods based upon conjugate addition of the entire ω-side chain, as an organometallic complex, to 2-alkylcyclopent-2-enones. Using this approach Sih and co-workers have achieved a completely stereo selective route to PGE_1 in which an organocuprate (e.g. 45) was added to a suitably protected 4-hydroxycyclopentenone (e.g. 46a) to give an intermediate (47) having the stereochemistry required at all centres of the prostaglandin molecule [50–51]. PGE_1



(45)



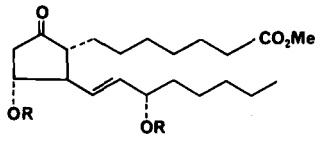
(46)

a. X = CH_2CH_2, R^1 = $Si(Me)_2Bu^t$, R^2 = Me

b. X = CH_2CH_2, R^1 = R^2 = H

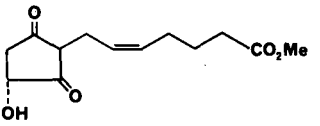
c. X = CH=CH, R^1 = Me, R^2 = H

d. X = CH_2CH_2, R^1 = Me, R^2 = H



(47)

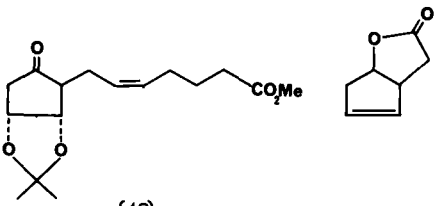
R = $Si(Me)_2Bu^t$



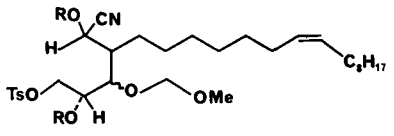
(48)

was then generated from (47) by acid hydrolysis of the alcohol protecting groups followed by microbial hydrolysis of the ester function. PGE_2 was synthesized in a similar manner based on the enone (46c). The enones (46) used for this work were synthesized by preparing hydroxydiketone (48), by microbial reduction of the corresponding cyclopentane trione, and then reduction of a C-3 carbonyl of a 1-enolate and an acid rearrangement.

Subsequently, several other routes to intermediates of this type have been published. Thus the hydroxyenone (46b) has been obtained by microbial hydroxylation of 7-(5-oxo-1-cyclopentenyl)heptanoic acid (2-(1-oxocyclopent-2-ene)heptanoic acid) [52]; the ether (46c) resulted from hydrolysis and dehydration of the dioxolan (49), prepared from the (+)-lactone (50) *via cis-*

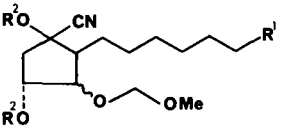


(49) (50)



(51)

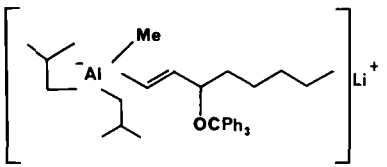
R=CH(Me)OEt



(52)

a. R^1=  , R^2=CH(Me)OEt

b. R^1=CO_2Me , R^2=H



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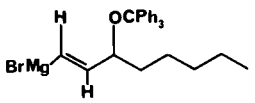


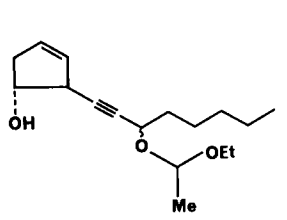
(54)

dihydroxylation, dioxolan formation and the usual Wittig elaboration *via* the lactol [53]; (46d) was prepared by constructing the cyanohydrin (51) from isopropylidene D-glyceraldehyde, cyclisation with sodium hexamethyl disilazane in benzene to (52a) and thence the sequence olefin cleavage, hydrolysis of ethoxyethyl, esterification to (52b), treatment with sodium hydroxide and acidification with hydrochloric acid in ether–tetrahydrofuran [54].

The alkenylalanate (53) [55] and the Grignard reagent (54) in the presence of copper I catalyst [56] have also been used for conjugate additions to enones of this type.

It has also been shown that reagents of type (45) in which the double bond is *cis* afford higher yields and greater stereoselectivity than the corresponding *trans* compounds [57]. The *cis* (15β) product prepared in this way could be isomerised to the required *trans* 15α product *via* a [2,3]sigmatropic rearrangement of a sulphenate ester to a 13-sulphoxide-14,15-*trans*-compound, and then treatment with trimethyl phosphite.

(c). Other approaches. Stork and Isobe [58,60] have reported a route to $PGF_{2\alpha}$ in which both side chains are built up via cyclopentadiene oxide. The ω-chain was attached first by reaction of the lithium salt of the 2-ethoxyethyl ether of 1-octyn-3-ol with cyclopentadiene oxide and the benzyl ether of the product (55) taken forward by three steps – hydrolysis of ethoxyethyl, partial reduction of the acetylene with lithium aluminium hydride and reprotection as the benzyloxymethyl ether – to the cyclopentene (56a). The enol phosphinate (56b) was then generated regioselectively from the latter by formation, with *N*-bromosuccinimide, of the bromohydrin (57), Jones oxidation and reaction of the resulting bromoketone with methyl diphenylphosphinite. Reaction of the enolate (56b) with *t*-butyllithium followed by treatment with zinc chloride and then formaldehyde afforded a 2-hydroxymethyl compound which underwent elimination of water on treatment of the mesylate with diisopropylethylamine to give the methylene cyclopentenone (58). Carbons 1–6 of the prostaglandin α-



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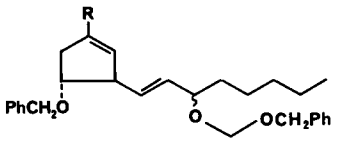
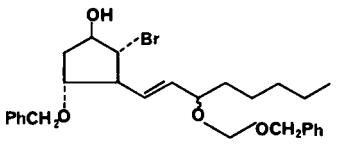


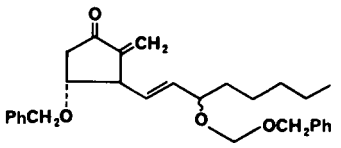
(56)

a, R = H

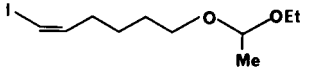
b, R = OP(O)Ph$_2$



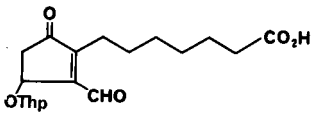
(57)



(58)



(59)

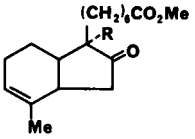


(60)

side chain were then attached by addition of the divinyl cuprate from the vinyl iodide (59) and the synthesis completed *via* hydrolysis of the ethoxyethyl protecting group with 50% acetic acid, Jones oxidation of the 1-alcohol to the carboxylic acid, reduction of the 9-ketone with tris-2-butyllithiumborohydride and removal of the benzyloxymethyl groups with sodium in liquid ammonia–ethanol to give (±)-$PGF_{2\alpha}$ and its C-15 epimer. Intermediates of type (58) have also been made *via* the thermal ene reaction of an acyclic enyne [60].

Full details of the Ciba-Geigy route which originally led to (±)-PGE_1 methoxime have now been published [1, p. 336; 61]. A noteworthy development of this work was the protection of the 9-ketone as the phenylthiomethyl oxime. This grouping was resistant to the mild oxidative and reductive stages of the synthesis and was cleaved with mercury ion catalysis to the unsubstituted oxime and thence by nitrosation to the free ketone yielding (±)-PGE_1.

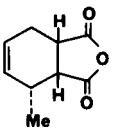
Miyano's earlier work on the synthesis of 15-dehydroprostaglandins [1, p. 342] has now been adapted to afford PGE_1 [62] by reducing the double bond in the cyclopentenone aldehyde (60) with chromous sulphate followed by Wittig elaboration. The earlier synthesis by Merck, Sharp and Dohme [1, p. 337] has now been published in full and several improvements effected in this approach [63–65]. The key intermediate (61a) has been synthesized by a more efficient route from the Diels–Alder addition product (62) (of *trans*-piperylene and maleic anhydride) which was transformed by homologation *via* reduction to the diol and reaction of the ditosylate with sodium cyanide to give the diacid (63). Iodolactonisation of the latter to (64a), esterification and deiodination to (64b), saponification of the ester lactone and dehydration of the corresponding dimethyl ester led to the cyclohexene (65) which afforded (61b) on cyclisation with ensuing alkylation and then (61a) by decarboxylation. Optical resolution



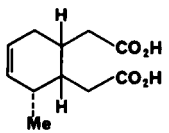
(61)

a . R = H

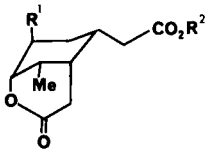
b . R = CO_2Me



(62)



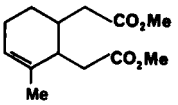
(63)



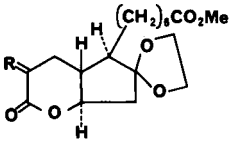
(64)

a . R^1 = I, R^2 = H

b . R^1 = H, R^2 = Me



(65)



(66)

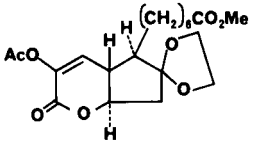
a . R = H_2

b . R = CHOH

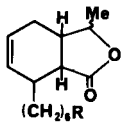
could be effected by conversion of (62) to a half methyl ester and forming the dehydroabietyl ammonium salt or by preparing the same salt of the acid corresponding to (64b).

Intermediate (61) has also been taken forward by a new procedure whereby the ethylene ketal, by olefin cleavage, esterification, Baeyer–Villiger oxidation and lactonisation, afforded (66a), of which the formyl derivative (66b) on ozonolysis and acetylation gave (67). PGE_1 then followed *via* olefin cleavage and Wittig elaboration [65].

Merck chemists [66] have also published a new route to (±)-PGE_1 based on the Diels–Alder products (68ab) of angelica lactone and the appropriate undecadiene which were converted *via* an oxidative cleavage and recyclisation to the cyclopentanones (69). The latter were then transformed into the acetal-ketal (70) which on saponification and oxidation with ruthenium dioxide–



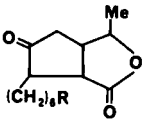
(67)



(68)

a, R = CO_2Me

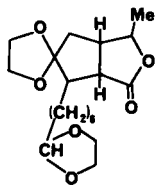
b, R = CH_2OAc



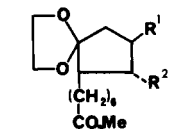
(69)

a, R = CO_2Me

b, R = CH_2OAc



(70)

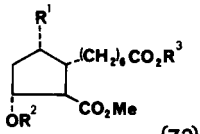


(71)

a, R^1 =  $R^2 = CO_2H$

b, $R^1 = OAc$, $R^2 = CO_2H$

c, $R^1 = CO_2H$, $R^2 = CH_2OThp$



(72)

a, $R^1 = CO_2H$, $R^2 = H$, $R^3 = Et$

b, $R^1 = COMe$, $R^2 = Ac$, $R^3 = Et$

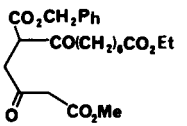
c, $R^1 = OThp$, $R^2 = Thp$, $R^3 = H$

sodium periodate yielded a glycollic acid. Treatment with sodium methoxide effected an ester exchange and epimerisation of the acetyl function to yield (71a), and (±)-PGE_1 then followed a Baeyer-Villiger transformation to (71b), reduct-

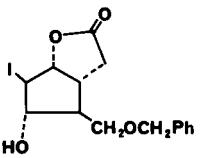
ion of the acylimidazole derivative of the carboxylic acid with lithium aluminium tri-*t*-butoxide to give the aldehyde and Wittig elaboration. Sankyo chemists [67] have described a route to (71c) from the ethylene glycol ketal of dimethyl cyclopentanone-3,4-*trans*-dicarboxylate. Sankyo workers [68] have also constructed the intermediate (72a) by alkaline cyclisation of (73) followed by catalytic reduction with hydrogenolysis and borohydride reduction. Acetylation and treatment of the acid chloride of the product with dimethyl copper lithium afforded (72b) and then Baeyer–Villiger oxidation and transesterification with potassium bicarbonate in methanol gave a diol of which the tetrahydropyranyl ether was subjected to selective ester hydrolysis to give the mono ester (72c). Reduction of the methyl ester with lithium borohydride to the primary alcohol and then, after re-esterification of the α-chain carboxyl group, elaboration of the ω-chain *via* the aldehyde led to (±)-$PGF_{1\alpha}$ methyl ester and thence (±)$PGF_{1\alpha}$. This approach has also been adapted to the synthesis of (±)-PGE_1 [69]. PGE_2 and $PGF_{2\alpha}$ have also been synthesized by transformation of the PGAs obtained from the Gorgonian coral *Plexaura homomalla* [70].

PROSTAGLANDINS A, C AND D

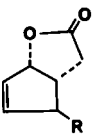
PGA. Several direct routes are now available to the A prostaglandins which were hitherto synthesized only by dehydration of the E compounds. Thus the Corey lactone approach has been adapted *via* elimination of the iodohydrin (74)



(73)



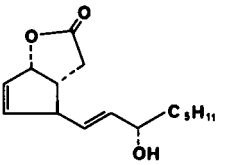
(74)



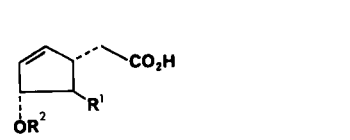
(75)

a, R= CH_2OCH_2Ph

b, R=CHO



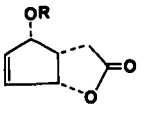
(76)



(77)

a, R^1 = $\wedge OCH_2CCl_3$ R^2 = H

b, R^1 = C_5H_{11} (OH) R^2 = $CONHC_6H_4\underline{p}Ph$



(78)

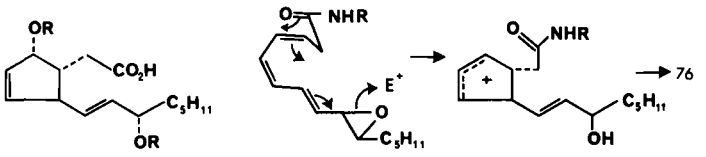
R = Dimethyl-t-butylsilyl

with methanesulphonylchloride to furnish the unsaturated lactone (75a) which was then elaborated to A prostaglandins *via* the aldehyde (75b) [71].

Two methods afforded routes to the intermediate lactone (76) which was also taken forward by the usual sequences. In one of these [72] the cyclopentene (79), prepared by peracid or peroxide cleavage of the appropriate bicyclo[2.2.1]-heptenone, was converted by a sequence which included a resolution stage, into the intermediate (77b). The latter then underwent cyclisation to the lactone (76) on heating in aqueous dimethoxyethane, a reaction in which the *p*-aryl-urethano unit afforded the driving force. In the other method [73], the lactone (78) was synthesized from the (±) lactone (50), *via* a resolution of the corresponding hydroxy acid and the ω-chain inserted by cross coupling of the vinylic copper reagent (45) to give carboxylic acid (79) which afforded (76) on cyclisation under acidic conditions.

A particularly interesting approach by Corey, Fleet and Kato [74] which resembles the biogenetic synthesis of prostaglandins, was achieved by means of the cyclisation of the epoxytrienamide (80) with boron trifluoride etherate and then hydrolysis with potassium biphthalate to afford the lactone (76) and the corresponding C-OH epimer.

An intramolecular cyclisation was also employed in a route from Kousse-Uclaf workers [75] whereby the pyrrolidine enamine of β-epoxy olefin (81) afforded the cyclopentanone (82a) by the action of sodamide. The latter, after alkylation of the Thp ether with ethyl 7-bromo-5-heptenoate to give (82b),

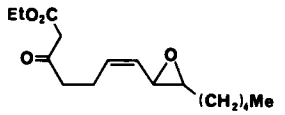


(79)

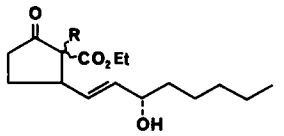
R = Dimethyl-t-butylsilyl

(80)

R = $\underline{p}$-C_6H_4Ph

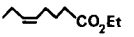


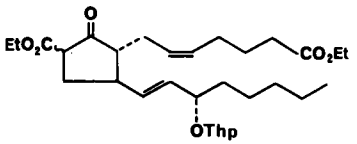
(81)



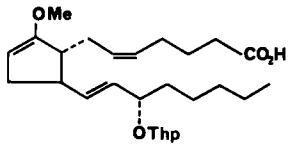
(82)

a, R = H

b, R = 



(83)



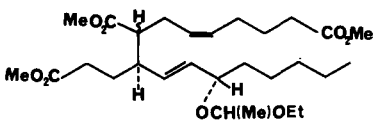
(84)

underwent a ring opening and recyclisation to the 11-deoxyprostaglandin (83) which afforded (±)-PGA_2 on reaction with diazomethane, saponification, decarboxylation, treatment of the resulting enol (84) with bromine and methanol followed by reaction with diazabicyclononene.

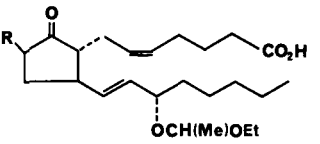
Stork and Raucher [76] have reported a chiral synthesis of PGA_2 starting from 2,3-isopropylidene-L-erythrose from which they prepared the intermediate (85). Cyclisation with potassium *t*-butoxide in tetrahydrofuran and alkaline hydrolysis then afforded the keto acid (86a) which was transformed into PGA_2 *via* reaction with lithium diisopropylamide and then phenyl selenyl chloride, oxidation of the product (86b) with sodium periodate and removal of the ethoxyethyl group.

PGC. Corey and Moinet have extended the prostaglandin A work to afford synthetic entry into the PGC series [77] by reacting the Thp ether of the lactol derived from the lactone (76) with triiron dodecacarbonyl to give the stable conjugated diene complex (87) which afforded PGC_2 on Wittig elaboration, Collins oxidation and hydrolysis. Jones in conjunction with Upjohn workers synthesised the aldehyde (88) by elimination of benzoic acid from lactone (1d) under mildly basic conditions which was then elaborated to PGC_2 methyl ester by the usual methods [78]. The Syntex team [79–81] also synthesized the Thp ether of PGC_2 from this aldehyde which was obtained by treatment of the acetoxy-aldehyde (1a) with methanolic potassium carbonate.

PGA_2 has also been transformed into PGC_2 on treatment with *t*-butoxide or



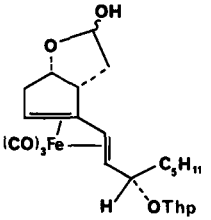
(85)



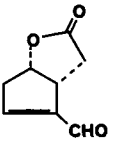
(86)

a, R = H

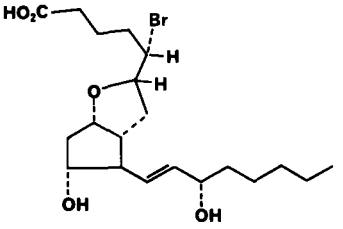
b, R = SePh



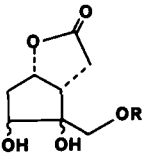
(87)



(88)



(89)



(90)

R = COC6H4p Ph

t-pentoxide ion and reaction of the enolate so obtained with acetic acid in methanol [82].

PGD. PGD_2 has been synthesized by Ono workers [83] *via* a low temperature oxidation with Jones reagent of the 11-hydroxy group of the 15-Thp ether of $PGF_{2\alpha}$ and by Ciba-Geigy [84], from Jones oxidation of the 9,15-di-Thp ether of $PGF_{2\alpha}$.

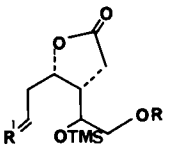
PROSTACYCLINS (PGI) AND THROMBOXANES

Two syntheses of the unstable prostacyclins have been announced, starting from $PGF_{2\alpha}$. Corey, Keck and Székely [85] achieved this transformation by reacting

the 11,15-bis-Thp derivative of $PGF_{2\alpha}$ with *N*-bromosuccinimide to give a diastereoisomeric mixture of bromo ethers which on depyranylation were separated and the major isomer (89) treated with potassium *t*-butoxide to effect elimination of hydrogen bromide. Rapid extraction of the product with ether and treatment with diazomethane then afforded the acid sensitive methyl ester of PGI_2. Samples of prostacyclin were obtained for bioassay as the pyrrolidine salt by prompt treatment of the above ethereal extract with pyrrolidine.

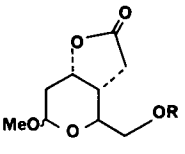
In the other route, by workers at the University of Pennsylvania and the Cardeza Foundation [86], the methyl ester of $PGF_{2\alpha}$ was converted by treatment with iodine in methylene chloride in the presence of potassium carbonate to an intermediate which was rapidly and almost completely converted to the methyl ester of prostacyclin by treatment with sodium ethoxide in ethanol. Prostacyclin was obtained as the sodium salt by the addition of water.

Several syntheses of thromboxane B_2 (TXB_2) have been published [87–92]. Two routes from Upjohn [87,88] were based upon cleavage of the cyclopentane ring in intermediates related to the Corey lactone (I). In one of these procedures [87] the glycol mixture (90) prepared from aldehyde (88) by borohydride reduction, *p*-phenylbenzoylation and hydroxylation was cleaved with paraperiodic acid and the resulting aldehyde-ketone reduced with sodium borohydride to give a mixture of diols of which the bis(trimethylsilyl ether) (91a) of the major isomer was subjected to a Collins oxidation to give the ketone (91b). The latter was then cyclised by treatment with methanolic hydrochloric acid to a mixture of acetals (92a). These were separated and each isomer (α and β OMe) taken forward by hydrolysis with sodium methoxide to the primary alcohols (92b) which were oxidised to the aldehydes and the side chains elaborated by the usual procedures to give 2 pairs of isomers (93ab and 93cd). Both (93b) and (93d) yielded TXB_2 on hydrolysis. In the second method [88] the cyclopentanone (94), obtained by Jones oxidation of the corresponding alcohol, was treated with *m*-chloroperbenzoic acid to give the lactone (95) of which



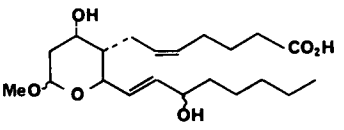
(91)

a, R = $COC_6H_4\underline{p}$ Ph, R^1 = H, OTMS
b, R = $COC_6H_4\underline{p}$ Ph, R^1 = O

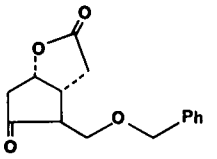


(92)

a, R = $COC_6H_4\underline{p}$ Ph
b, R = H
c, R = ^{t}BDPSi

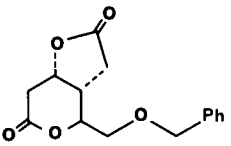


(93)

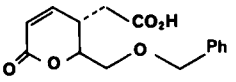


(94)

a, α-OMe(15R)*
b, α-OMe(15S)
c, β-OMe(15R)
d, β-OMe(15S)
*Prostaglandin numbering



(95)

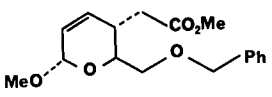


(96)

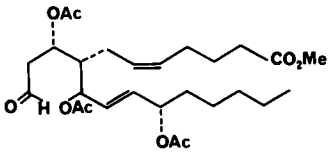
the elimination product (96), from base treatment, afforded lactone (92b) (α-methoxy isomer) on ketone reduction with diisobutylaluminium hydride, reaction with diazomethane and hydrogen chloride in methanol and triethyl orthoformate, and then sequential treatment of the major product (97) with sodium hydroxide, carbon dioxide and potassium iodide and iodine followed by deiodination of the resulting iodolactone and debenzylation.

In a third Upjohn synthesis [89] the 9,15-diacetoxy derivative of $PGF_{2\alpha}$ methyl ester underwent ring opening with lead tetraacetate to the triacetoxy aldehyde (98) of which the dimethyl acetal afforded TXB_2 after basic hydrolysis of acetate and ester groups and then hydrolysis of the acetal with phosphoric acid in aqueous tetrahydrofuran.

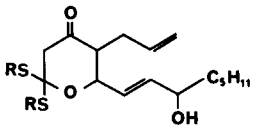
Corey, Shibasaki, Knolle and Sugahara [90] constructed the intermediate (99) by lithiation of the enone (100a), prepared by reaction of the lithium enolate of the readily available enone (100b) with allyl bromide, and reaction of



(97)

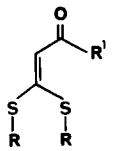


(98)



(99)

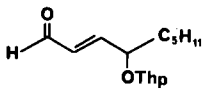
R = nC_4H_9



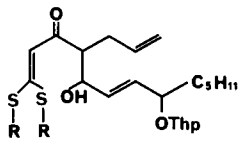
(100)

a, R = $n C_4H_9$, R^1 = $(CH_2)_2CH{=}CH_2$

b, R = $n C_4H_9$, R^1 = Me



(101)

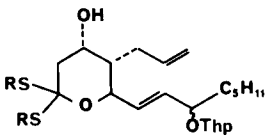


(102)

R = $n C_4H_9$

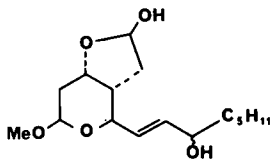
(100a) with the aldehyde (101) to give the adduct (102) as a diastereoisomeric mixture which afforded (99) on depyranylation and acid catalysed cyclisation in methylene chloride. The Thp ether of (99) was then reduced by borohydride to a mixture of diastereoisomeric alcohols of which the required product (103) (15α/β epimer mixture, prostaglandin numbering) was hydrolysed with silver nitrate–silver oxide to the corresponding hydroxylactone. The latter was treated successively with diisobutylaluminium hydride and methanol containing boron trifluoride etherate and the resulting hydroxy acetal subjected to dihydroxylation and cleavage of the diol to give the lactol-acetal (104) which was converted to (±)-TXB_2 and its C-15 epimer by the usual methods.

Hanessian and Lavallee [91] started from the readily available *ribo*-hexopyranoside (105) of which the benzoate was sequentially hydrogenolysed and silylated with *t*-butyldiphenylsilyl chloride to give the alcohol (106a). Oxidation

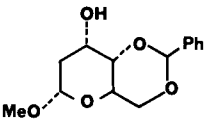


(103)

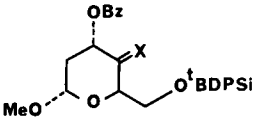
R = $n C_4H_9$



(104)



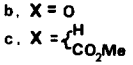
(105)

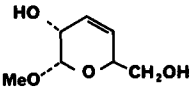


(106)

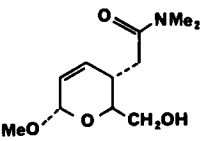
a. X = 

b. X = O

c. X = 



(107)



(108)

to the ketone (106b) and Wittig elaboration then afforded a mixture of two products (106c) which gave lactone intermediate (92c, α-methoxy isomer) on hydrogenation and treatment with carbonate in methanol. The ester (92c) was then taken forward to TXB_2, in this instance the α-chain being attached prior to the β-chain.

In another approach, Corey, Shibasaki and Knolle effected a stereospecific Claisen rearrangement of the unsaturated sugar (107) by reaction with the dimethylaminal of *N,N*-dimethylacetamide in diglyme, to give the dimethylamide (108). The latter then afforded (92b) (α-methoxy epimer) on reaction with iodine and deiodination of the resulting iodolactone with tributyl tinhydride [92].

PROSTAGLANDIN ANALOGUES

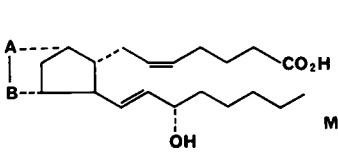
In recent years a major proportion of the papers on prostaglandin chemistry has been devoted to the synthesis of prostaglandin analogues and many other modified prostaglandins are disclosed in the now voluminous prostaglandin patent literature. This largely reflects the extensive efforts devoted by the pharmaceutical companies to the synthesis of compounds which might exhibit advantageous features compared with the naturally occurring prostaglandins, such as more potent and selective biological activity, longer duration of action, greater chemical stability and simplicity of structure.

Here an attempt is made to indicate briefly the main trends in this work.

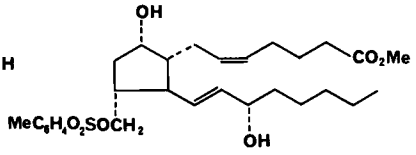
Because of the large number of analogues prepared it has been necessary to confine description of synthetic methods to a few selected types, including those which, because of their interesting properties, form the subject of discussion in the biological sections of this review. In any case, many of the analogues are prepared by relatively straightforward adaptations of the general routes to the natural prostaglandins described in preceeding sections. For convenience the analogues are grouped as follows: (a) replacement of the rings; (b) modifications at C-9 to C-11; (c) α-chain analogues and (d) the ω-chain analogues; many of the compounds synthesized incorporate a combination of more than one of these features.

(a) Replacement of the rings. Analogues have been prepared in which the cyclopentane ring has been replaced by cyclohexyl [93] cyclobutyl [94], and by a variety of heterocyclic systems. The latter include 8-, 10- and 12-aza [95–97], 9-, 10- and 11-oxa [98–100] and 9- and 11-thia [101,102] compounds in which one of the cyclopentane ring carbons is replaced by a heteroatom, as well as analogues where the ring has been replaced by aromatic heterocyclic rings e.g. furan, oxazole and thiazole [13, p. 66; 103].

Ring replacement has assumed a particular importance with the bicyclic endo-



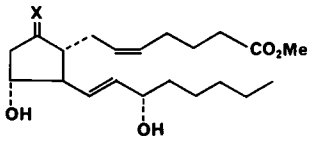
(109)



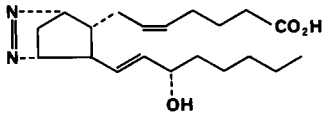
(110)

a, A = O, B = CH_2

b, A = CH_2, B = O



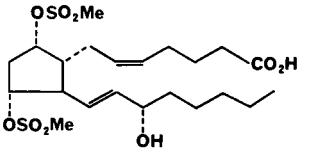
(111)



(112)

a, X = H, CH_2OSO_2Me

b, X = CH_2



(113)

(114)

peroxide prostaglandins because the instability of the peroxide linkage generally precludes working with the natural compounds. Analogues have been synthesized in which the peroxide in PGH has been replaced by other linkages which are more stable but at the same time preserve the rigid geometry of this bicyclic ring system. Upjohn have prepared two cyclic ethers (109) in which a methylene group has been substituted for each one of the peroxide oxygens [104]. Compound (109a) was made by an intramolecular cyclisation with potassium hydroxide in aqueous methanol of the tosylate (110) which had been synthesized from PGA_2 by a benzophenone-sensitised photoaddition of methanol to give 11-deoxy-11-hydroxymethyl-PGE_2, reduction to the corresponding $PGF_{2\alpha}$ analogue, esterification and tosylation of the primary alcohol.

The other cyclic ether (109b) was synthesized by a similar cyclisation of the mesylate (111a), the latter being prepared by reaction of the 11,15-bis(trimethylsilyl ether) of PGE_2 methyl ester with *N*-methylphenylsulphonimidoylmethyl magnesium chloride, reductive elimination of the resulting β-hydroxysulphoxime intermediate with aluminium amalgam to give the 9-methylene compound (111b), conversion to the 11,15-triphenyl silyl ether and then selective hydroboration of the exo-methylene to afford the primary alcohol; mesylation and silyl ether hydrolysis.

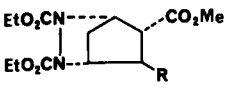
Harvard workers synthesized the 9,11-aza analogue (112) of PGH_2. This was prepared initially by reaction of the bismesylate (113), prepared in four steps from the 10,11-epoxide of PGA_2, with hydrazine and then aerial oxidation of the resulting cyclic hydrazide in the presence of copper acetate [105].

More recently a total synthesis of (112) has been achieved from the Diels–Alder adduct (114) of diethyl azodicarboxylate and methyl cyclopentadienecarboxylate [106]. Conjugate addition to 114 of nitromethane, conversion of the nitromethyl product to the aldehyde (115a) and elaboration by the usual methods then led to a diastereoisomeric mixture of allylic Thp ethers (115b). The latter was then hydrolysed to the acid with potassium hydroxide under carefully selected conditions, converted by sequential treatment with ethyl chloroformate-triethylamine and sodium borohydride to the corresponding alcohol and the latter treated with potassium hydroxide under more vigorous conditions to remove the two ethoxycarbonyl groups. The resulting free hydrazine was directly oxidised by air in the presence of cupric acetate catalyst to give the azo

compound (116a) and the synthesis continued by oxidation with chromium trioxide in pyridine to the aldehyde followed by Wittig reaction with methoxymethylenetriphenylphosphorane to give the enol ether (116b). The free aldehyde of the latter, generated under neutral conditions (using mercuric acetate as catalyst followed by treatment with potassium iodide) afforded (112) on Wittig elaboration, conversion to the methyl ester, Thp cleavage, separation of epimers and ester hydrolysis.

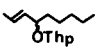
Corey has also synthesized a bicyclo[2.2.1]heptene analogue of PGH, in which the peroxide is replaced by a vinylene grouping [107].

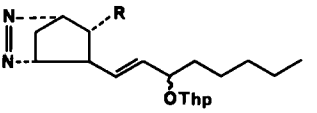
(b) Modifications at C-9 to C-11. The chemical instability of the PGA, C and



(115)

a, R = CHO

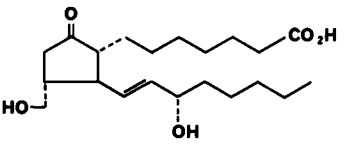
b, R = 



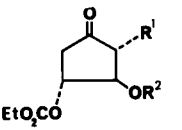
(116)

a, R = CH_2OH

b, R = CH=CHOMe



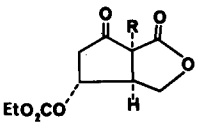
(117)



(118)

a, R^1 = H, R^2 = CO_2Et

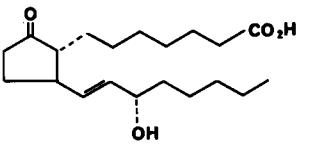
b, R^1 = $(CH_2)_6CO_2Me$, R^2 = H



(119)

a, R = H

b, R = $(CH_2)_6CO_2Me$



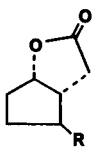
(120)

E prostaglandins has prompted the synthesis of more stable analogues. Thus compounds e.g. 8-methyl-PGC_2 [108], 12-methyl-PGA_2 [109] and (±)-10, 10-dimethyl-PGE_1 [110] have been synthesized having methyl groups in key positions which block the normal transformation to the relatively inactive PGB products.

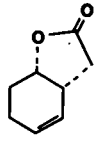
Many other ring substituted prostaglandins have been prepared with the object of discovering compounds with interesting activity. Among the few compounds of this group known to exhibit a high level of biological activity are the 11-deoxy-11-hydroxymethylprostaglandins from the Sankyo Laboratories [111,112]. 11-Hydroxymethyl-PGE_1 (117) was synthesised *via* the cyclopentanone (118a) which was converted with potassium *t*-butoxide to the lactone (119a). Alkylation of the latter to (119b) and then hydrolysis and decarboxylation led to (118b) which was elaborated to (117) *via* the usual methods.

The corresponding 11-β-hydroxymethyl compound [113], as well as the 12-hydroxymethyl- [114], 9-hydroxymethyl- [115], and 9,11-dihydroxymethyl- [116], analogues have also been prepared and other ring substituted analogues include 10-hydroxy- [117,118], 10-hydroxy-11-deoxy- [119,120], 8-methoxycarbonyl- [121] 10,11-methylene- [122] 10,11-ethylene- [123], 10- and 11-methyl- [124–126]. Many 11-substituted prostaglandins have also been synthesized from prostaglandins obtained from the Gorgonian Coral source [127].

The 11-deoxyprostaglandins [1, p. 341] have attracted considerable attention and several new synthesis have been described. Although these compounds are generally less potent biologically than the natural prostaglandins the absence of the C-11 hydroxyl renders them available in fewer steps, and with the E series (e.g., 11-deoxy-PGE_1, 120), free from the inherent instability from which the natural E compounds suffer as a result of their readiness to undergo dehydration. New syntheses include adaptation of the Corey lactone approach, thus the

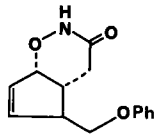


(121)

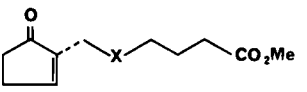


(122)

a. R = CHO
b. R = CH_2OPh
c. R = CH_2OH



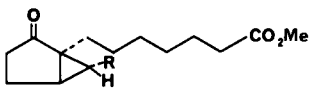
(123)



(124)

a , X = $-CH_2CH_2-$

b , X = $-CH \overset{cis}{=} CH-$

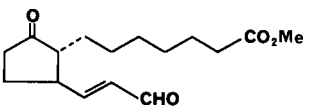


(125)

a , R = CO_2H

b , R = CHO

c , R = $CH-CH_2$ (O)



(126)

key intermediate lactone aldehyde (121a) has been prepared from the cyclohexene (122) by cleavage with thallium III nitrate and perchloric acid–sodium perchlorate and recyclisation [128], or from the iodohydrin [129]. Intermediate (121b) has been prepared by a thermal nitrogen extursion from the cyclic hydroxamic ester (123), the latter being made from a nitro-bicyclo[2.2.1]-hept-5-ene intermediate by an abnormal Nef reaction [130]. Alcohol (121c) has been obtained *via* reduction of the dimethyl ester of *trans* β-carboxy-α-carboxymethyl cyclopentanone [131].

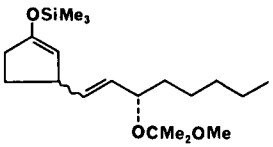
The enones (124a, b) have been used extensively in the synthesis of 11-deoxyprostaglandins, the ω-chain being added by the organometallic complex method as in the synthesis of the natural prostaglandins [e.g. 132–135] or by the 1,4-addition of nitromethane, conversion of the resulting nitromethyl compound to the aldehyde by a Nef-type reaction, thence proceeding *via* the usual Wittig elaboration [136,137].

Melnikova, Grigorev and Pivnitsky [138] obtained the bicyclic compound (125a) as the major product from addition of dimethylsulphuranylidene acetate to (124a). Transformation *via* reduction to the alcohol, oxidation to the aldehyde (125b), Wittig elaboration and epoxidation was followed, after saponification, by reductive ring opening with lithium in liquid ammonia and

then re-esterification and oxidation to give the keto aldehyde (126) which afforded (±)-11-deoxy-PGE_1, via Grignard elaboration.

Patterson and Fried [139] inserted the α-side chain by alkylation of a cyclopentatone enol ether. Intermediate (127) was first obtained by reaction of cyclopent-2-enone with an organocopper reagent followed by formation of the silyl enol ether and the α-chain then added by alkylation of 127 with *cis*-7-bromooct-5-enoate, thus leading to (±)-11-deoxy-PGE_2 methyl ester.

In a synthesis by the May and Baker team [124] the ω-hydroxyheptyl cyclopentenone (128) was prepared by condensation of cyclopentanone morpholine enamine with 7-hydroxyheptanal and isomerisation of the resulting



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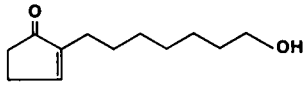

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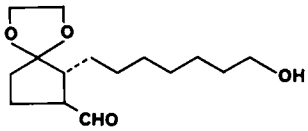

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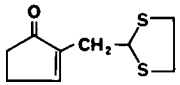

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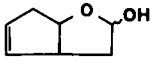

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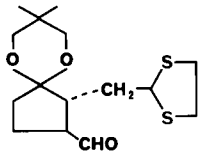


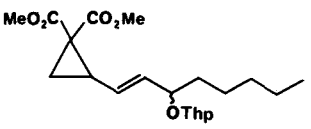
(132)

hydroxyalkylidene cyclopentanone by heating with hydrochloric acid in butan-1-ol, and elaborated by conjugate addition of nitrile, ketal formation, conversion with diisobutylaluminium hydride to the hydroxyaldehyde (129) and then the sequence Wittig reaction, alcohol oxidation with chromium trioxide–sulphuric acid in dimethylformamide, ketone reduction and ketal hydrolysis to give (±)-11-deoxy-PGE_1 and the corresponding C-15-epimer.

Hoechst workers [140] used steps similar to those employed in the (128) to (129) transformation to convert the enone (130), prepared from the hemiacetal (131) by thioacetal formation, Moffat oxidation and olefin bond migration, to the aldehyde (132) which was then elaborated to afford (±)-11-deoxy-PGE_2.

In a synthesis of optically active 11-deoxy-PGE_1, Abraham [141] prepared the cyclopropane derivative (133) by ozonolysis of the methyl ester of (-)-2-vinylcyclopropane-1,1-dicarboxylic acid and elaboration of the resulting aldehyde, and then effected a condensation with trimethyl heptane-1,1,7-tricarboxylate to give, after hydrolysis, the cyclopentanone (134) which afforded 11-deoxy-PGE_1 and its C-15 epimers on alkali treatment.

The methoxy bicycloheptadienone (135) was obtained [142] by irradiation of α-tropolone methyl ether which, after hydrogenation of the conjugated double bond, underwent cleavage by ozonolysis of the enol ether double bond to give the dimethyl acetal (136). The latter was then alkylated with ethyl 7-



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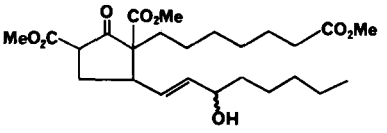

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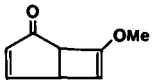

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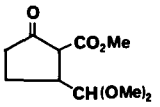

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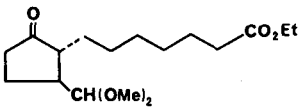

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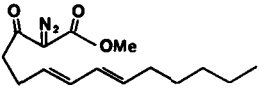
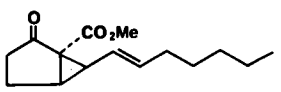


(138)

iodoheptanoate and decarboxylated with sodium cyanide in hexamethylphosphoric triamide to give (137) which was then hydrolysed to the aldehyde and elaborated to (±)-11-deoxy-PGE_1.

Chemists at the Sagami Research Centre constructed the bicyclic compound (139) by thermolysis of (138) in benzene in the presence of copper acetoacetate. Ring opening of (139) with potassium thiophenoxide in *t*-butanol led to (140) which was elaborated to (±)-11-deoxy-PGE_1 *via* alkylation with methyl 7-iodoheptanoate and decarboxylation to provide the α-chain, and then successive treatment with *m*-chloroperbenzoic acid and trimethyl phosphite [143]. 9-Deoxyprostaglandins have also been synthesized [144–146].

(c) α-Chain modifications. Many variants of the α-chain have been incorporporated into prostaglandin analogues. The terminal portion of the chain has received particular attention by insertion of groupings such as 3-oxa- [13, p. 873; 147] and *trans* olefin [148,149] which may modify activity as a result of blocking β-oxidation, one of the metabolic inactivating pathways of the prostaglandins. Modifications have also been made in the 5,6- position where the double



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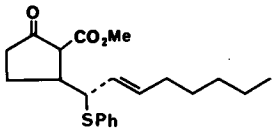

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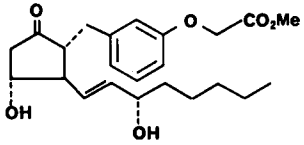

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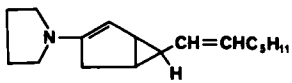

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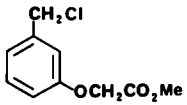

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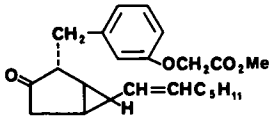
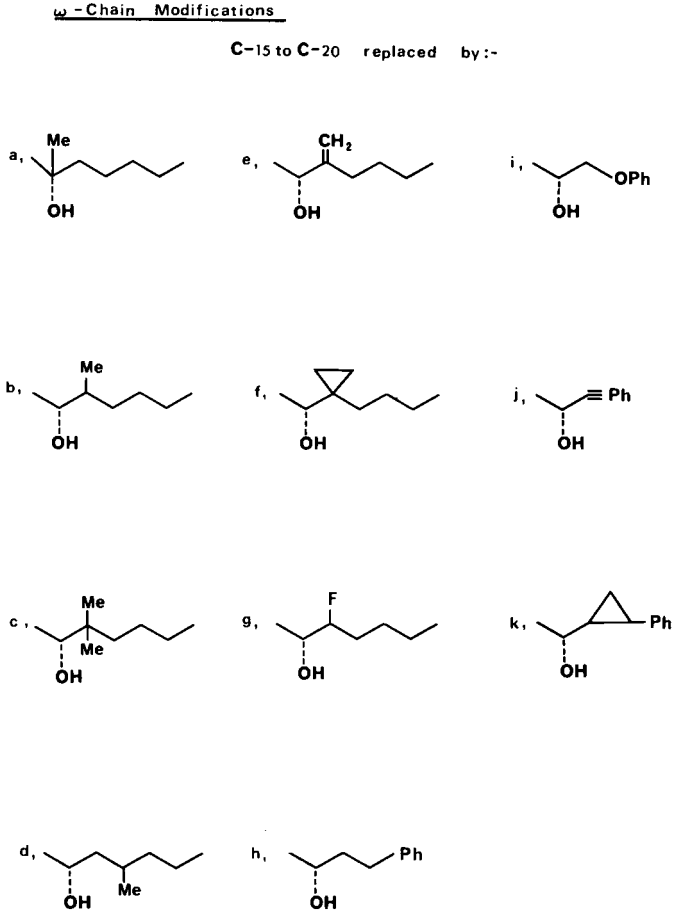


(144)

bond of the '2' series has been replaced by an acetylene [150] or by an interphenylene grouping [151], the latter compounds (e.g. 141) being synthezied by reaction of the enamine (142) with the halo ester (143) and elaboration of the product (144) by the general procedure used previously for transformation of bicyclohexane intermediates of this type [1, p. 332]. Other α-chain analogues prepared include 4- and 5-oxa- [141], 2-carboxy- [152], 2-decarboxy-2-sulphonic acid [153], 2-decarboxy-2-(tetrazol-5-yl)- [154], 7-oxo- [155], and various unsaturated derivatives [149,156].

(d) ω-Chain modifications. Modification of the ω-chain has been by far the most successful area in producing analogues having greater potency and selectivity of biological actions than the naturally occurring prostaglandins. Several of



(145)

the analogues e.g. 15- and 16-alkyl have been designed to block the metabolic inactivation of the C-15 hydroxy group by prostaglandin dehydrogenase. Groupings which have conferred particularly interesting activity include 15-methyl- (e.g., 145a) [157–159], 16-methyl- (e.g., 145b) [160–162], 16,16-dimethyl- (e.g. 145c) [161,162], 17-methyl- (e.g., 145d) [13, p. 781], 16-methylene- (e.g., 145e) [163], 16,16-ethano- (e.g., 145f) [163], 16-fluoro- (e.g., 145g) [164], 17-phenyl- (e.g., 145h) [165], 16-phenoxy- (e.g., 145i) [166], 15-phenylalkynyl- (e.g., 145j) [167] and arylcyclopropyl (e.g., 145k) [163].

The synthesis of these compounds was relatively straightforward, the 16- and 17- substituted compounds being prepared by reaction of the appropriate Wittig type reagent with Corey lactone aldehyde intermediates of type 1 and the C-15 methyl compounds *via* a Grignard reaction of an appropriate ketone.

The effect of the position of the hydroxy group in the ω-chain has been studied in a series of analogues which were prepared by adaptations of the organometallic complex method (p. 370) [168–170]. Other ω-chain analogues which have been prepared include variations in the double bond region to afford 13,14-dehydro- [171,172], 13,14-dihydro-13,14-methylene- [173] and 14-chloro- [172], as well as various alkoxy- [174] and ω-homo- [175] compounds.

In addition to the above structural analogues, syntheses have been reported of unnatural epimers and enantiomers of the natural prostaglandins [176,177].

BIOCHEMISTRY AND METABOLISM

It is no longer possible, nor even desirable, to review the progress made in broad areas of prostaglandin research covered by the general headings of biochemistry, pharmacology, physiology and therapeutic applications. The pace at which advances have been made in our understanding of the naturally occurring prostaglandins and related compounds is far too rapid to be adequately covered by one review and quite beyond the capacity of any one scientist to catalogue. In an effort to help the specialised reader of this review, an indication is given at the beginning of each of the following sections of some of the reviews on individual aspects of prostaglandin research which are, in our opinion, worthy of reading for additional information. All the reviews quoted contain comprehensive bibliographies, and they cover some of the more important recent developments in much more detail than is possible in the present work.

There are a number of reviews and books written since the last one in this series [1] dealing with the biosynthesis of prostaglandins, and those published even as late as 1976 [5–12,15,16] describe a relatively straightforward sequence of biochemical transformations as illustrated in *Figure 8.2* for the formation of PGE_2 and $PGF_{2\alpha}$ from arachidonic acid. Since many original references are well

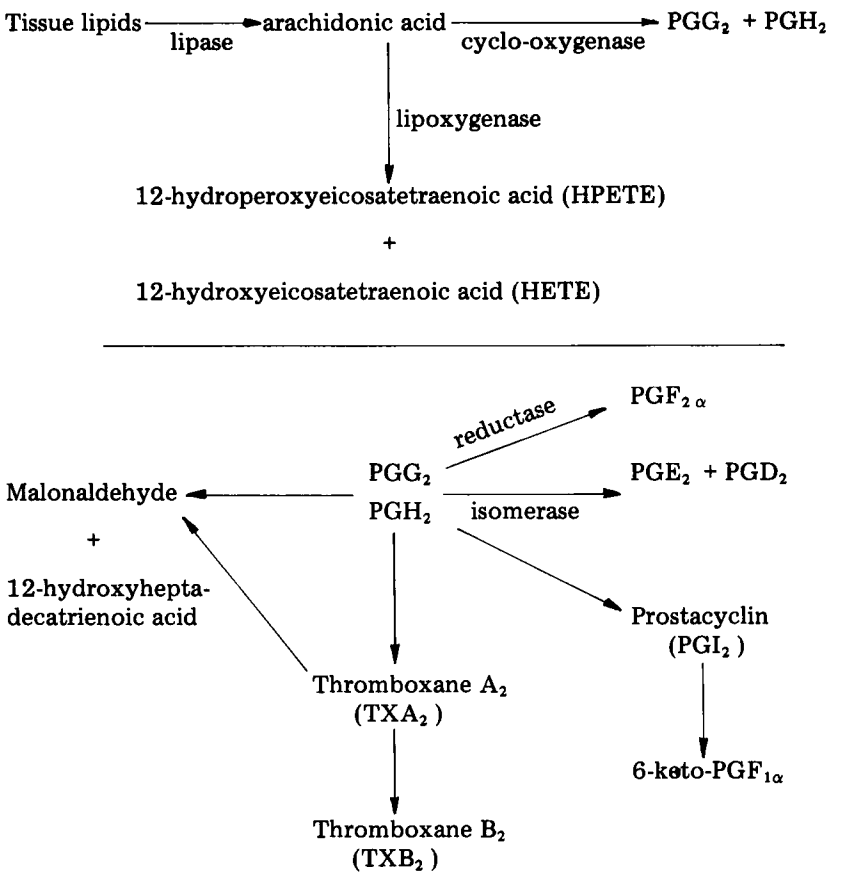


Figure. 8.2. Abbreviated scheme for the biochemical transformation of the prostaglandin endoperoxides PGG_2 and PGH_2 to other products (see Figure 8.1)

documented in these previous reviews, they are not included in the present chapter. In 1973, two independent groups reported the isolation of the unstable endoperoxide intermediates PGG_2 and PGH_2 [178,179] which had been proposed originally by Samuelsson in 1965 [180]. The isolation of these intermediates, which were prepared by incubation of arachidonic acid and a microsomal fraction of a homogenate from the vesicular gland of sheep, represented a substantial technical achievement since these endoperoxides are very unstable in aqueous solutions and PGG_2 has a half-life of about 30 min at 20° [179]. When stored in dry non-polar organic solvents, the stability of PGG_2 is improved with a half-life at 37° of 2.7 h and at −70° it can be stored for

several weeks. Preliminary data reported in these papers revealed that these endoperoxides were substantially more potent than the natural prostaglandins E_2 and $F_{2\alpha}$ subsequently formed from both PGG_2 and PGH_2 by the enzymes endoperoxide isomerase and endoperoxide reductase respectively.

In a subsequent publication [19], the Swedish group reported improved methods of biosynthesis and purification of both PGG_2 and PGH_2 and additional biological data. PGG_2 was reported to be 50–200 times more potent than PGE_2 on the superfused rabbit aorta strip and PGH_2 was 100–450 times more potent than PGE_2 on the same preparation. In addition, both endoperoxides were found to be potent stimulators of platelet aggregation. Exciting though these developments were, they were only a prelude to even more revolutionary disclosures.

An international conference on prostaglandins was held in Florence, Italy on May 26–30, 1975 and the majority of the participants were immediately rewarded by an introductory lecture by Samuelsson who presented his recent data on the isolation and identification of two new products isolated from platelets which were given the names thromboxane A_2 and B_2 [13, pp. 1–6] in recognition of the potent platelet aggregation properties of thromboxane A_2. The structures of these new compounds and the pathways for their formation from arachidonic acid are shown in *Figure 8.3*. A report of this work together with additional new data was presented in two succeeding publications [181,182]. Yet again, a key feature of this work was the ability of the Swedish workers to isolate the extremely unstable thromboxane A_2 ($T_{1/2}$ = 30–40 s) and to carry out a preliminary evaluation of its pharmacological properties. An oxane ring structure containing an unstable epoxide (*Figures 8.1 and 8.3*) was proposed for thromboxane A_2 by Samuelsson as being the most likely structure leading to the formation of the stable thromboxane B_2. This latter compound was shown to be relatively devoid of biological activity, although by analogy with the more intensively studied prostaglandins, this view may eventually prove to be erroneous since the 'inactive' PGB compounds have recently been shown to have potent pulmonary bronchoconstrictor properties [183].

However, even these fundamentally important and exciting findings have been overshadowed by the very recent discovery by a group at the Wellcome Research Laboratories in England, of yet another short lived, extremely potent prostaglandin intermediate. This material, formed from PGG_2 or PGH_2 by an enzyme isolated from arterial smooth muscle [4], was initially described as a new prostaglandin and named PGX, but which has more recently been designated as PGI_2 [20]. It is the most potent inhibitor of platelet aggregation yet discovered in the prostaglandin family of compounds and a potent coronary and arterial dilator. Its structure was elucidated in a joint effort by the Wellcome team and Upjohn scientists [184]. The structure of PGI_2 and its biosynthetic pathway from PGG_2 is shown in *Figure 8.4*. The most interesting feature of this

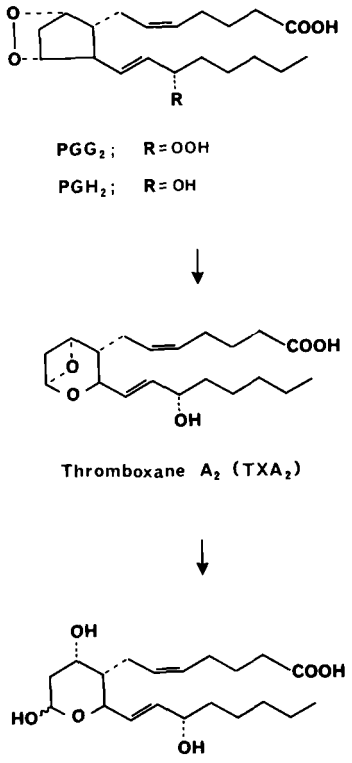


Figure 8.3. Transformation of prostaglandin endoperoxides to thromboxanes

work is the demonstration that the short-lived PGI_2 is converted to 6-keto-$PGF_{1\alpha}$, a compound which was previously isolated from challenged guinea-pig lung [185] and from stomach homogenates by Pace-Asciak [186] and has since been isolated from rabbit heart [187] and from rat, guinea-pig and sheep uteri [188]. In view of the biochemical evidence indicating that 6-keto-$PGF_{1\alpha}$ is the stable end-product of PGI_2 formation, this suggests that there could be substantial prostacyclin formation in heart, lung, uterine and arterial tissue from a number of species. The biological significance of the prostacyclins will be discussed in later sections.

One of the most consistent and rewarding aspects of the many fundamental findings by Vane and his co-workers for the past ten years has been their use of bioassay procedures in the characterisation and isolation of novel, biologically active compounds from isolated perfused organs, tissue preparations or enzyme

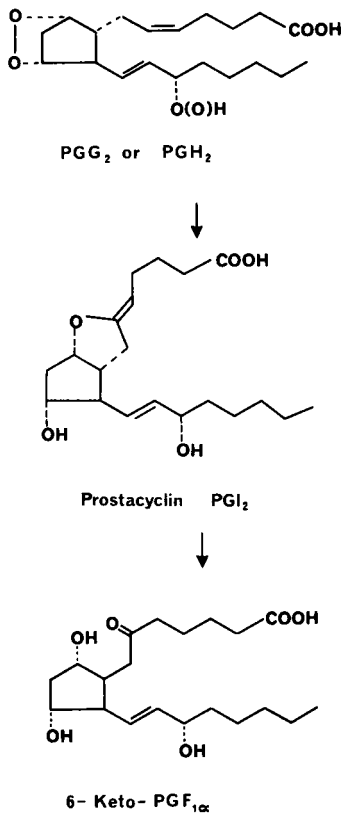


Figure 8.4. The formation of PGI_2 and 6-keto-$F_1\alpha$ from PGG_2 [9]

preparations [189]. It was by the use of these techniques that the potent but short lived activity of prostacyclins was discovered [4] and previously Piper and Vane [190] had described the release of an unstable rabbit aorta contracting substance (RCS) from guinea-pig lungs. The isolation and identification of the unstable endoperoxides PGG_2 and PGH_2 led to suggestions that RCS may be a mixture of the endoperoxides. However, it was found that although the endoperoxides were active on rabbit aorta, their half-life was considerably longer than that of RCS. It has recently been shown that the more unstable and major component of rabbit aorta contracting substance formed in both platelets and guinea-ig lung is thromboxane A_2 [3].

In 1971, Pace-Asciak and Wolfe reported the isolation and identification of a new prostaglandin derivative formed from arachidonic acid by rat stomach homogenates [191]. This compound (I, *Figure 8.5*) was postulated to be formed

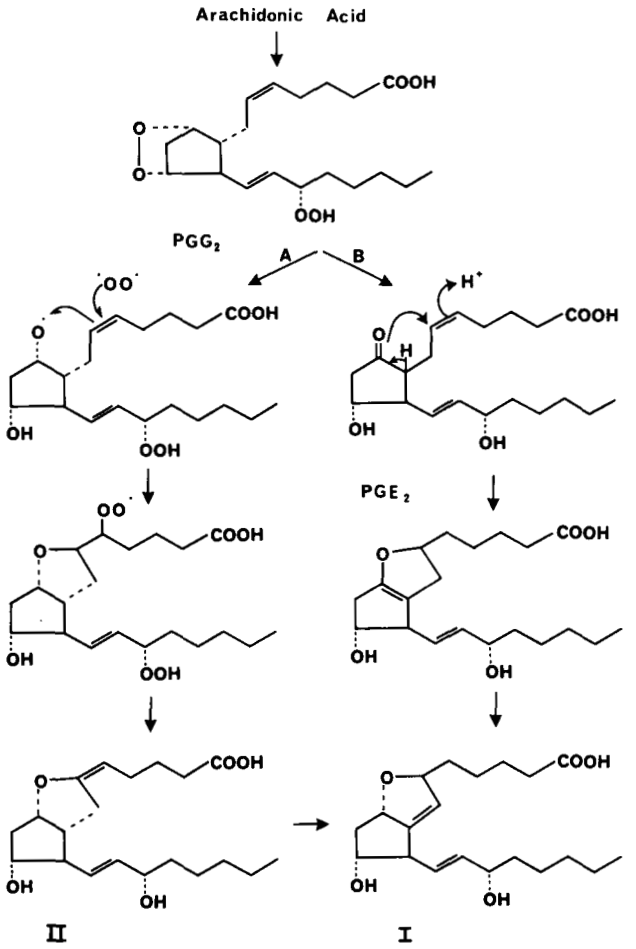


Figure 8.5. Two pathways (A and B) proposed by Pace–Asciak and Wolfe in 1971 [16] for the formation of compound I from arachidonic acid in rat stomach homogenates. The proposed intermediate II is PGI_2

from one or both of the endoperoxide intermediates PGG_2 and PGH_2 by the proposed pathways A or B. In the course of this work, compound II was identified as a minor component of the incubation products and this was considered to be suggestive evidence for pathway A. We now know compound II is the same as PGI_2, and had these workers, back in 1971, carried out the same range of pharmacological assays on the products isolated from rat stomach homogenates as did Vane and his co-workers for the characterisation of prostacyclin formed

from rabbit aortas, then there is little doubt that our knowledge of these fascinating compounds would have been advanced much earlier.

The isolation and identification of these relatively unstable and potent intermediates have led a number of scientists to question the physiological importance of the primary prostaglandins of the E and F series. In many organs far larger concentrations of thromboxanes and 6-keto-$PGF_{1\alpha}$ (indicative of PGI_2) are found compared with prostaglandins E_2 or $F_{2\alpha}$. In addition, the biological activity of the biosynthesized endoperoxides PGG_2 and PGH_2 seems to be much more important than that of PGE_2 and $PGF_{2\alpha}$, and in other organs the formation of the hydroxylated fatty acids HETE and HHT (*Figure 8.2*) could have important physiological consequences. These observations, which will be discussed in more detail in later sections, have led to suggestions [192] that PGE_2 and $PGF_{2\alpha}$ are not always the most fundamentally important products of arachidonic acid metabolism but often the first, albeit biologically active, metabolites of endoperoxide formation, just as thromboxane B_2 and 6-keto-$PGF_{1\alpha}$ are metabolites of thromboxane A_2 and PGI_2 respectively.

There are a number of comprehensive reviews of PGE_2 and $PGF_{2\alpha}$ metabolism [193,194]. Two groups have recently reported that the major urinary metabolite of circulating thromboxane B_2 in the cynomolgus monkey is the dinor compound [195,196]. Both groups were unable to find any appreciable concentrations of products expected to be derived from oxidation of the alcohol group at C-15 by prostaglandin 15-hydroxydehydrogenase (EC 1.1.1.141, PGDH) or reduction of the Δ^{13} double bond by the appropriate reductase.

Research into the pharmacological properties and physiological importance of the primary prostaglandins has continued at an ever increasing pace. There is no doubt now that the prostaglandins of the E series (PGE_1, PGE_2 and PGE_3) and of the F series ($PGF_{1\alpha}$, $PGF_{2\alpha}$ and $PGF_{3\alpha}$) are local hormones or autacoids. That is, they are biologically active compounds, synthesized by a variety of tissues and organs and acting locally on those organs. After exerting their local action, the bulk of them are metabolised by the enzymes 15-hydroxyprostaglandin dehydrogenase and prostaglandin $\Delta^{13,14}$-reductase to biologically inactive metabolites at their site of synthesis and action, e.g., in the cortex, brain, GI tract including stomach and liver [1,5]. In certain organs, considerable quantities of prostaglandin are released into the venous effluent of that organ, e.g. kidney [197] and spleen [198] and these are extensively metabolised on passage across the lungs [199]. Furthermore, all the biochemical evidence accumulated to date leads us to believe that prostaglandins are not stored in organs after synthesis, but are continuously synthesized and released, albeit in varying concentrations depending on the stimuli responsible for controlling their synthesis. There are two possible exceptions to the concept of prostaglandins being local hormones. The first arises from the technical fact that platelets are an efficient source of

continuously synthesized prostaglandins [86, pp. 293–343; 200] and thus platelet derived prostaglandin can be considered as a form of circulating hormone able to act on a number of other organs and tissues.

The second exception arises from the well documented observations that PGA_1 and PGA_2 are relatively resistant to the action of PGDH [201] and in fact a large proportion of circulating PGA_2 (administered as an exogenously administered intravenous injection) escapes destruction on passage across the lung [199]. However, one of the other properties of a hormone is that it must be synthesized by an organ and transported to another organ where it has a site of action. In the case of the PGA compounds, there has been a long-standing controversy concerning the natural occurrence of these compounds *in vivo*. This arises from the fact that both PGE_1 and PGE_2 are readily dehydrated by non-enzymatic processes during their isolation and purification from tissues or fluids. Thus PGA_2 was first identified in extracts of rabbit renal medulla [202] although evidence was obtained indicating that some or all of this material could have been derived from PGE_2, the major prostaglandin present in rabbit renal medulla. Biochemical experiments failed to demonstrate any biosynthesis of PGA_2 from arachidonic acid in rabbit renal medullary preparations [203]. More recently, Fröhlich, Sweetman, Carr and Oates have demonstrated conclusively that there is no naturally occurring PGA_2 in rabbit renal medulla [204] or in human plasma [205]. There is a recent review of this subject which discusses these findings in some detail [8c, pp. 1–39]. Likewise it was accepted for many years that considerable quantities of PGA_1, PGA_2, 19-hydroxy-PGA_1 and 19-hydroxy-PGA_2 were present in human seminal plasma [206]. However, it has now been shown that these compounds all arise by dehydration of the naturally occurring PGE and 19-hydroxy PGE compounds in seminal plasma [207].

PHARMACOLOGY

PRIMARY PROSTAGLANDINS

The pharmacology of prostaglandins of the E, F and A series (*Figure 8.1*) has been studied extensively since the last report in this series [1] and present readers can refer to a number of admirable reviews of this work for further information [5–9, 11c, 15,16]. These reviews deal with various aspects of prostaglandin pharmacology, but there is little doubt that one of the earliest reviews written by Bergström, Carlson and Weeks [208] and a more recent one by Weeks [209] provide the best general coverage.

There has been considerable effort devoted to finding a common factor associated with the biological activity of prostaglandins. There are numerous reports

indicating that prostaglandins of the E series can profoundly affect the levels of cyclic AMP in a variety of tissues, leading to associated changes in cellular Ca^{2+} concentrations. This work has been the subject of a conference which was held in 1972 and the proceedings have been published [210]. Goldberg has also documented the importance of intracellular cyclic GMP concentrations [211] and it has been shown that $PGF_{2\alpha}$ can cause large increases in cGMP concentrations in rat uterus [12, pp. 155–172]. These observations have led a number of investigators to propose that prostaglandins could have a physiological role as intracellular messengers, and the evidence has been reviewed [212].

General reviews which also include substantial discussion of prostaglandin pharmacology are also available [213–217]. The proceedings of a meeting held to discuss kinins and prostaglandins cover many pharmacological aspects [218], and there are reviews on prostaglandins in cutaneous biology [219] and on prostaglandins and the digestive system [220].

SYNTHETIC PROSTAGLANDINS

The naturally occurring prostaglandins have a wide variety of pharmacological actions capable of affecting in one way or another just about every organ of the body. However it is unrealistic to expect that all the prostaglandins synthesised by medicinal chemists could be screened for all their pharmacological properties in a routine manner. In general, a few key pharmacological screens are employed to detect activity which would be likely to yield a potential product for a specific therapeutic indication. In the case of prostaglandin analogues, these are most generally screened for luteolytic potency, abortifacient, hypolipidaemic and hypotensive properties, as inhibitors of platelet aggregation, and for efficacy as bronchodilator, anti-secretory and anti-ulcer agents. All these properties are potentially useful for therapeutic development. However prostaglandins are also capable of producing severe side effects so they also have to be tested for their potential to produce diarrhoea and vomiting, central nervous system side effects including headaches, hypotension or other related vasoconstrictor effects, and constriction of pulmonary airways leading to bronchoconstriction. In addition, a number of desired therapeutic effects could not be developed if the compound had other properties, normally classed as desirable, but incompatible with the primary use. Thus an effective anti-ulcer prostaglandin could not be developed if it was also an abortifacient or a potent hypotensive agent at doses similar to that producing the desired therapeutic effect.

For these reasons, it is probable that a great number of potent and effective prostaglandin analogues which have been the subject of detailed pharmacologic studies, reports and even preliminary clinical trials will never proceed to

pharmaceutical development. Very often, these side effects can be predicted during pharmacological screening and no further studies will be required. However there are no simple animal screens for nausea or headache, and even simple tests of vomiting in dogs are unreliable indicators of potency because dogs are particularly susceptible to numerous prostaglandin analogues at doses which do not cause vomiting in other species.

The prostaglandin analogues which are discussed in this and later sections of this review have been selected from the many reported in the literature as being those which have been the subject of clinical evaluation or which have a combination of potency and selectivity in animals which suggest that they could prove to be therapeutically useful.

One of the earliest and still one of the most interesting series of analogues to be studied in some detail includes the 15(*S*)-15-methyl-PGE_2 compound and its methyl ester (145a, *Figure 8.6*) [12, pp. 76–90; 157,158,221] and 16,16-dimethyl-PGE_2 and its methyl ester (145c, *Figure 8.6*) [160,161]. The methyl esters of these two compounds were reported to be potent inhibitors of gastric acid secretion by Robert and Magerlein [12, pp. 247–253], being some 50–100 times more potent than the naturally occurring PGE_2 in inhibiting gastric secretion of acid and pepsin and in preventing the formation of peptic ulcers in laboratory animals. There have since been numerous reports substantiating the activity of these two compounds [222–225] in laboratory animals. This last report [225] summarises all the work carried out by the Upjohn group and also provides a very useful review of references to other studies carried out on these analogues. It has also been reported that 15(*R*)-15-methyl-PGE_2 methyl ester is also a potent anti-secretory agent [226,227]. An early review by Karim and Ganesan which covers the effects of prostaglandins on the digestive system [220] provides a good introduction to this area of research.

The potent anti-ulcer actions of these synthetic analogues, especially against ulcers produced by non-steroidal anti-inflammatory agents, have been discussed by Robert [228]. In view of the numerous reports of gastrointestinal side effects following treatment of patients with analgesic and anti-inflammatory agents such as aspirin and indomethacin, Robert has suggested that the combination of a non-steroidal anti-inflammatory drug with a prostaglandin analogue might prevent the occurrence of these gastrointestinal side effects [228] and more recently Robert has described this property as a cytoprotective effect [8c, pp. 225–266].

The biological activity [229,230], pulmonary effects [231] and cardiovascular actions [232] of the 15(*R*)-15-methyl analogue of PGE_2 have been reported. In addition, both 15(*S*)-15-methyl-PGE_2 methyl ester and 16,16-dimethyl-PGE_2 have been reported to be potent inhibitors of hormone-stimulated lipolysis [233], and the potency and selectivity of these analogues on rat gastro-

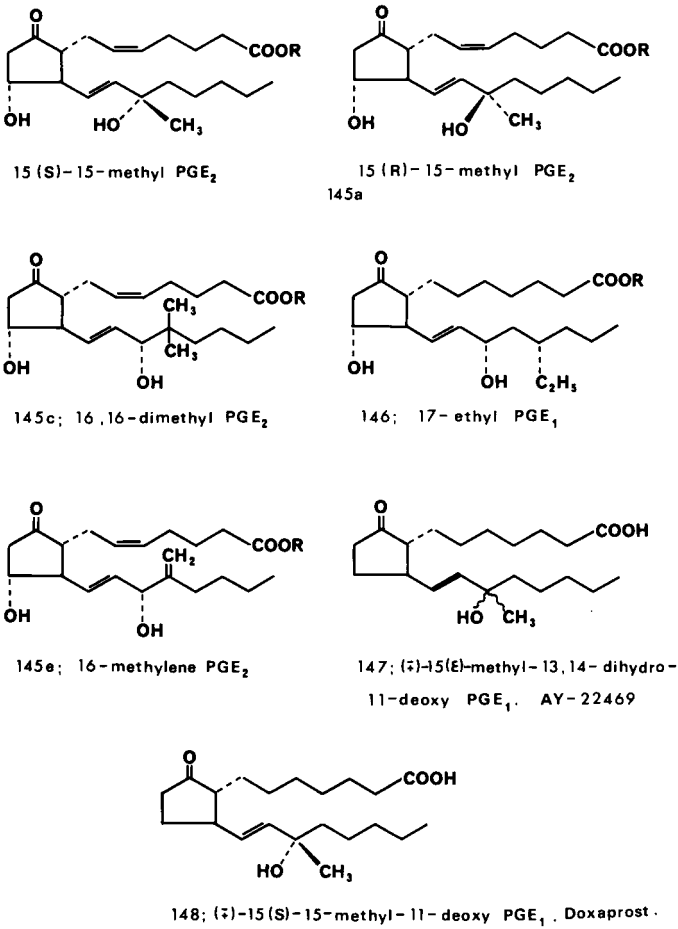


Figure 8.6. 15-, 16- and 17-substituted prostaglandin analogues with important biological properties. R=H or Me

intestinal function have been compared [234]. In general, these analogues are 40–100 times more potent as anti-secretory and anti-ulcer agents than PGE_2, but they are also more potent diarrhoea agents which suggests that they may not have an acceptable selectivity of action in man.

Another series of analogues, based on the 11-deoxy-PGE_1 [120] and PGE_2 structures, have been studied in considerable detail by a number of groups. The potential advantages enjoyed by these compounds arises from the fact that they are more stable than the natural series (PGE_1 and PGE_2 readily dehydrate to

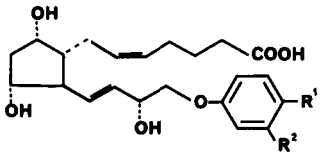
PGA and PGB compounds, *Figure 8.1*) and they are also relatively inactive diarrhoeal agents. The first report of the anti-secretory action of 11-deoxy-PGE_1 [120] was by Lippmann who reported that this material (AY-20,524) was one-tenth as active as PGE_1 in the Shay rat test [235] when administered sub-cutaneously. The same worker has also reported that a mixture of the two 15-hydroxy-15-methyl enantiomorphis of (±)-9-oxoprostanoic acid (i.e., (±)-15-(ξ)-methyl-13,14-dihydro-11-deoxy-PGE_1; AY-22,469) had oral anti-secretory activity in this test [236] and oral anti-ulcer activity in a pylorus-ligated rat test [237] in contrast to the simple analogue 15(ξ)-hydroxy-9-oxoprostanoic acid (i.e. (±)-13,14-dihydro-11-deoxy-PGE_1; AY-22,093) which was inactive in these tests. Furthermore (±)-AY-22,469 had an anti-secretory ED_{50} value of 4.3 mg/kg (p.o.) compared with a diarrhoea ED_{50} value of 120 mg/kg (p.o.) in rats. When the more widely used indomethacin-induced ulcer rat test was used [239] Lippmann found that a large number of 11-deoxy-PGE_1 analogues were active, but the most potent compounds were AY-22,469 and its $\Delta^{13,14}$ analogue (15(ξ)-methyl-11-deoxy-PGE_1), both compounds being 12 times less potent than PGE_2. Interestingly, the anti-secretory and anti-ulcer potencies of AY-22469 [240] were found to be lower than 15(nat)-16,16-dimethyl-11-deoxy-PGE_2 (AY-24,609) [241]. For example, AY-24,609 was 5 times more potent than AY-22,469 in the rat indomethacin test. Thus although little comparative data of these compounds and PGE_2 have been published it appears that even the most potent analogue in this series is about half as potent as PGE_2. Since PGE_2 has been reported to have no anti-secretory activity at a dose of 40 μg/kg (p.o.) in man [242], it is probable that substantially higher doses of the 11-deoxy analogues will have to be administered clinically to produce anticipated anti-secretory and anti-ulcer effects. There have been no reports of such clinical trials to date. Comparable data with that of Lippmann have been reported recently by Caton and Crowshaw [162] for a range of structurally similar 11-deoxy-PGE_1 analogues. Another interesting analogue based on the PGE_1 molecule, in which the C-15 hydroxy group is transposed to the C-16 position has been reported to have good oral activity [243]. A more active derivative of this compound is (±)-15-deoxy-16α,β-hydroxy-16-methyl-PGE_1 methyl ester (SC-29333), being more potent than PGE_1 in the dog. It is also long acting and orally active [244].

Many prostaglandins of the E_1 series including 11-deoxy PGEs are potent bronchodilators. The problems associated with the screening of bronchodilator prostaglandin analogues in animals [162] have been discussed and these problems can make it difficult to obtain accurate assessments of potency relative to PGE_1 or PGE_2. The Ayerst analogue AY-22,093 referred to above has been reported to be a bronchodilator qualitatively similar to PGE_2, having a direct effect on smooth muscle but less potent than PGE_2 [245]. The Ayerst workers

have recently described the synthesis of (±)-15-(ξ)-methyl-11-deoxy-PGE_1 and its separation to the two C-15 epimers. The more potent natural isomer (148, *Figure 8.6*) has been given the name Doxaprost [246] and in a series of comparative bronchodilator tests (anaesthetised guinea-pig) against AY-22,093, Doxaprost was shown to be 73 and 32 times more potent by the aerosol and intravenous routes [247]. The activity of a series of 11-deoxy analogues including Doxaprost has been reported by Hall and Jaitly [248] and there is an excellent overall summary of the action of these 11-deoxy prostaglandins and related analogues on the respiratory tract of animals by Rosenthale, Dervinis and Strike [13, pp. 477—493]. Interestingly, a number of 15-methyl-11-deoxy-PGE_2 analogues have been synthesized by Wyeth chemists, and three were reported to induce platelet aggregation *in vitro* [249]. The most potent of these, Wy-17,186 (15(ξ)-methyl-11-deoxy-PGE_2) also exerts a haemostatic effect on a bleeding wound surface, a property which has potential clinical applications.

A large number of 15-methyl substituted enantioners of PGE_2 and $PGF_{2\alpha}$ have been studied for their anti-fertility properties in the pregnant hamster. 15(*S*)-15-Methyl-$PGF_{2\alpha}$ was the most potent analogue, being 100 times more active than $PGF_{2\alpha}$ in this test [250]. The synthesis of a number of 17-phenyl-18,19,20-trinorprostaglandins of the E_1 [251] and E_2 [252] series has been described and the biological properties of these compounds reported [253]. The most interesting of these Upjohn compounds appears to be 17-phenyl-18,19,20-trinor $PGF_{2\alpha}$ which was reported to be 90 times more potent than $PGF_{2\alpha}$ in the hamster antifertility assay and was active at a dose of 3 mg (i.m.) as a luteolytic agent in cows (as indicated by oestrus induction) compared with a dose of 25 mg $PGF_{2\alpha}$ (i.m.) required to produce a similar effect [253]. The metabolism of this 17-phenyl $PGF_{2\alpha}$ analogue has been studied in the cynomolgus monkey and in women, and the plasma and urinary metabolites identified [254]. A number of 15-keto metabolites were identified indicating that this compound is a substrate for 15-hydroxyprostaglandin dehydrogenase (PGDH).

The anti-fertility properties of a number of ICI prostaglandin analogues have been reported. An early compound in this series was ω-dihomo-$PGF_{2\alpha}$ [255] which was reported to be 5 and 20 times more potent than $PGF_{2\alpha}$ by the s.c. and oral routes respectively in terminating early pregnancy in hamsters. Using the same test and a comparable rat test, the activities of three novel ring-substituted 16-phenoxy-$PGF_{2\alpha}$ analogues (*Figure 8.7*) were determined [256] and shown to be at least 100 times more potent than $PGF_{2\alpha}$ [256]. The synthesis and biological activity of these compounds have been described in further detail [257,258] and more recently a number of related 16,17-configurationally rigid-17-aryl-18,19,20-trinor prostaglandins have been studied [259]. Two of these compounds, Estrumate and Equimate (*Figure 8.7*) are now marketed as veterinary agents for the regulation of oestrus in cattle and horses respectively.



ICI 79,939 $R^1 = F$, $R^2 = H$
ICI 80,996 Estrumate $R^1 = H$, $R^2 = Cl$
ICI 81,008 Equimate $R^1 = H$, $R^2 = CF_3$

Figure 8.7. Potent 16-phenoxy $PGF_{2\alpha}$ analogues active as luteolytic agents

The pharmacology of a number of the above analogues has been reviewed by Karim and Adaikan [11c, pp. 327–360] and the practical application of some of these analogues in animal husbandry has been discussed by Cooper and Walpole [11b].

STRUCTURE–ACTIVITY CONSIDERATIONS

The many types of biological activity exhibited by the prostaglandins offered early hopes that they would find eventual clinical application in a number of therapeutic areas. However the range of their biological activities also constitutes the major difficulty in achieving this objective, since successful medical application clearly demands specific activity only in the area required. Success in developing the prostaglandins for medical use is therefore largely dependent upon the discovery of prostaglandin analogues which are sufficiently selective in action to produce the required response with only minimal side effects at the therapeutic dose. Adequate duration of action and, in most cases, oral efficacy are also required.

Virtually the only rationale which offered itself as a guide to analogue synthesis was the blocking of known pathways of prostaglandin metabolism in the hope that this might product compounds with longer duration of action. It is significant therefore that the 15-methyl compounds (145a) (in which the deactivation mechanism by the primary inactivating enzyme, prostaglandin 15-hydroxydehydrogenase is totally blocked) as well as other analogues (e.g., 145c) which are known to be poor substrates for this enzyme, all showed enhanced activity compared with the corresponding naturally occurring prostaglandin. This is also true of the 16-phenoxy $PGF_{2\alpha}$ analogues (*Figure 8.7*) which have been shown to be poor substrates for PGDH [257,258]. However the potency

of these compounds is so high that there must be other factors operating which lead to enhanced activity and which are at present poorly understood, at least to these reviewers.

Modifications to the other parts of the molecule appear to have been made largely on an empirical basis and very few have been reported as showing significant improvements in desirable biological properties. The interphenylene compound (141) [151] was found to be 10 times as potent as PGE_1 in inhibiting collaginase–induced platelet aggregation in human platelet–rich plasma and had only weak smooth muscle activity [151].

Study of the effect of the position of the α-chain hydroxy group [168–170] led to the discovery that moving the 15-hydroxy group to C-16 produced an improvement in antisecretory properties and at the same time practically eliminated the unwanted smooth muscle stimulating action [243]. Further displacement to the 17 position caused almost complete loss of activity in both areas. There is also a dramatic increase in potency when an additional 16-methyl substituent is introduced [244].

11-Deoxyprostaglandins, although less active than the corresponding natural prostaglandins have the advantage of relative stability and ease of synthesis. These analogues, when combined with other features in the ω-side chain (15- and 16-methyl substituents) have an interesting level of activity and selectivity.

Although these developments show that chemical modification of the prostaglandin molecule can produce markedly different spectra of activity from the parent compounds, the position has not yet been reached in which it is possible to arrive at an overall assessment of the underlying principles governing structure activity. For many of the analogues synthesized, biological data are either not available or have only been reported very briefly; even where such information has been published it is often not possible to make direct comparisons between analogues because of the different test systems used. From what is known, it seems clear that the structure–activity pattern in the prostaglandins is a complex one, which might be expected for molecules of such flexible structure capable of assuming many different conformations. Attempts have been made to rationalise structure-activity findings in terms of physicochemical parameters and it has been postulated that the type of activity is governed by the relative dispositions of the side chains.

There have been two recent reviews dealing with structure–activity relationships. The earlier one by Anderson and Ramwell [15, pp. 30–50] discusses mainly the various epimers and isomers of the natural prostaglandins, whereas the more recent review by Schaaf [260] provides a concise discussion of the structure–activity relationships of a variety of analogues together with a comprehensive bibliography which should be referred to for further information in this area.

ENDOPEROXIDES PGG_2 AND PGH_2

After the first reports of the isolation of these endoperoxides from seminal vesicle preparations [178,179] which provided material for preliminary pharmacological evaluation, further studies demonstrated that platelet microsomal enzymes were able to synthesize large quantities of endoperoxides [261,262]. These synthetic endoperoxides are potent inducers of platelet aggregation and they are also potent stimulators of smooth muscle being 4 to 8 times more potent than PGE_2 on isolated rabbit aorta strips [178]. The pharmacological evaluation of these agents has been restricted to some extent by their difficulty of synthesis and by their relative instability. However two recent methods which provide simple and reliable syntheses of milligram quantities of PGG_2 and PGH_2 have been reported [263,264], and it is to be expected that there will be a corresponding increase in reports of pharmacological activity for these potent compounds.

Stable analogues of the endoperoxides have already been synthesized. The two cyclic ethers (109a and b) [104] and the aza analogue (112) [105,106] have been studied by a number of workers. In their original paper the Harvard workers reported that this aza analogue was substantially more potent (about 7-fold) than PGG_2 in stimulating isolated rabbit aorta strips and mimics PGG_2 and PGH_2 in their ability to induce platelet aggregation and release serotonin when added to human platelet-rich plasma. Malmsten has compared the biological properties of all three analogues in the same tests and reported that compound (109b) was the most potent [265]. The same three analogues have also been shown to be potent systemic pressor agents and powerful pulmonary vasoconstrictors [266], the cyclic ether endoperoxide analogues (109a and b) being twice as potent as the aza compound (112). Other workers have demonstrated the great potency of (109b) in increasing pulmonary arterial pressure [267,268]. All three compounds induced contractions of the smooth-muscle structures in the lungs and airways, whereas they were actually weaker agents than PGE_2 and $PGF_{2\alpha}$ on gastrointestinal smooth-muscle organs [269]. The property of these analogues may reflect the activity of the naturally occurring endoperoxides, which are in general difficult to elucidate since, as discussed previously (*Figure 8.2*), the endoperoxides are rapidly converted enzymatically to a number of potent compounds.

THROMBOXANES TXA_2 AND TXB_2

The unstable TXA_2 is a very potent compound formed from the endoperoxides PGG_2 and PGH_2 in a number of organs and it is rapidly inactivated to TXB_2

(*Figures 8.1* and *8.3*). TXA_2 is a potent inducer of platelet aggregation [13, pp. 19–28 and 737–746; 270] and a potent vasoconstrictor [271–273]. It is now clear that the compound released from guinea-pig lungs and described by Piper and Vane [273] as rabbit aorta contracting substance (RCS) consists mainly of TXA_2 with PGG_2 and PGH_2 as minor constituents [270]. The presence of TXA_2 in brain [274] and in homogenates of polymorhponuclear leukocytes [275] suggests that it may have important functions in the CNS and in inflammation.

PROSTACYCLINS

PGI_2 (*Figure 8.4*) is another important compound generated from the endoperoxides PGG_2 and PGH_2 [4,20] and it was found to be produced from these added precursors by blood vessel walls of pig, rabbit and man [276]. PGI_2 is a potent inhibitor of platelet aggregation and a vasodilator, especially of the coronary arteries, properties opposite to those of the parent endoperoxides. These properties led Vane [276] to suggest that the presence of an enzyme in the healthy vascular wall which converts platelet endoperoxides to the potent anti-aggregating substance PGI_2 explains why platelets do not adhere to normal vascular endothelium. This association of prostacyclin synthetase and the endothelial cells could explain why the initiation of a mural thrombosis, particularly in the arterial tree, is associated with endothelial damage. At this site, decreased prostacyclin synthesis from the platelet derived endoperoxides would lead to a lowered anti-aggregation effect and the increased concentration of endoperoxides would also be pro-aggregatory. However, prostacyclin formation by adjacent undamaged endothelium would limit the growth of the thrombus to the damaged area, in agreement with previously observed findings. Thus prostacyclins, or more stable analogues of a prostacyclin, hold promise as potent and effective anti-atheroma agents. In addition, a recent report which shows that PGI_2 is the major prostaglandin released from the isolated perfused rabbit and rat heart [277] suggests that prostacyclins may be essential, naturally occurring cardiac vasodilators, continuously produced by the heart to protect the coronary circulation against the formation of blood clots. Thus prostacyclins may be key substances whose biological properties could be important in the origin as well as in the prevention of coronary disease.

RECENT CLINICAL DEVELOPMENTS

PRIMARY PROSTAGLANDINS

There have been numerous reports of the administration of naturally occurring prostaglandins to volunteers and patients. Much of the early work is well

summarised in a review by Hinman [278] and in a more recent review by Karim and Hillier which related both to the pharmacology and clinical application of prostaglandins [279]. In addition there is a wealth of information in the published proceedings of a symposium held in South Africa [280]. These general reviews cover a number of potential and actual clinical applications, the two main ones being in obstetrics and gynaecology and in anti-ulcer therapy. The various clinical applications are summarised below.

Obstetrics and gynaecology. Readers who wish to become acquainted with the physiological aspects of the activity of prostaglandins in the reproductive system should read three reviews which cover the subject in some detail [281–283]. The ability of a number of prostaglandins especially $PGF_{2\alpha}$ [12, pp. 645–650] to affect the corpus luteum of a number of laboratory and farm animals and to cause a related decrease in progesterone release (luteolysis) has been utilised in the veterinary field. $PGF_{2\alpha}$ has been marketed as an agent for the regulation of oestrus in cattle and horses. However, no such luteolytic property has been reported in primates and women, even though $PGF_{2\alpha}$ can terminate pregnancy in women, and causes an associated interruption of corpus luteum function. This effect is probably mediated indirectly through the oxytocic properties of prostaglandins.

For the purposes of the present work, it is not possible to review in detail all the clinical work which has been carried out with the natural prostaglandins, especially since there are many excellent reviews of this subject already [5, pp. 167–199; 8a, pp. 347–389; 8b, pp. 175–203; 8b, 14; 15, pp. 77–84]. There is also a very readable account of the current status of prostaglandins as abortifacients [2]. Currently both PGE_2 and $PGF_{2\alpha}$ are sold commercially in many countries of the world for the induction of labour. There have been investigations into a variety of routes of administration, but one of the most remarkable observations to arise from clinical investigations was that orally administered PGE_2 was effective in inducing labour in women at term. This effect, since confirmed many times, was unexpected because of the known metabolism of prostaglandins by the gastrointestinal tract and, after systemic absorption, by the lungs and other major organs of the body as exemplified by the studies of Magee, Armour and Miller [285]. The major limitation to a more widespread use of natural prostaglandins in obstetrics and gynaecology is the relatively large number of common side effects including nausea, vomiting and diarrhoea which occurs to both PGE_2 and $PGF_{2\alpha}$ administered by a number of different routes. A good review of use of PGE_2 and $PGF_{2\alpha}$ in the induction of labour is by Barden [8c, pp. 109–133]. One of the objectives of current pharmaceutical research is to find analogues of these prostaglandins with a better selectivity of action.

Anti-secretory and anti-ulcer prostaglandins. Although intravenous admin-

istration of PGE_1 and PGA_1 has been shown to decrease stimulated gastric acid secretion in man, oral injestion of PGE_1 by volunteers failed to affect either the volume of gastric secretions or concentration of gastric acid. This work together with good background descriptions of the effects of prostaglandins on the gastrointestinal tract has been reviewed [5, pp. 287–323; 8, pp. 225–266; 11c, pp. 247–276; 15, pp. 112–118]. Despite the lack of oral anti-secretory activity, Fung and Karim [286] have carried out a small double-blind trial of oral PGE_2 in ulcer patients and shown that this prostaglandin is significantly better than placebo in promoting the healing of gastric ulcers. Although this work has not yet been confirmed by other workers, additional studies are in progress and confirmatory results could result in an additional therapeutic indication for oral PGE_2.

Bronchodilator prostaglandins. Aerosol doses of PGE_1 administered to healthy volunteers caused coughing and occasional retrosternal soreness [287] but when the PGE_1 was reformulated in an aerosol as its triethanolamine salt and administered to five subjects with reversible airways obstruction, bronchodilatation was observed. One of these subjects found the inhalation of PGE_1 to be irritant to the upper respiratory tract, but no coughing was reported [288]. However, reports of retrosternal soreness were commonly encountered, and in the one subject above, the irritant effect caused a slow but progressive fall in the FEV_1 and wheezing and bronchospasm was observed. PGE_2 has also been reported to have similar bronchodilator properties [288], whereas $PGF_{2\alpha}$ is a potent bronchoconstrictor [289] in healthy volunteers. Other workers have since reported that asthmatic subjects are 8,000 times more sensitive to the bronchoconstrictor effects of $PGF_{2\alpha}$ than healthy controls [290], leading the authors to suggest that $PGF_{2\alpha}$ may be one of the bronchoconstrictor factors in asthma. The side effects encountered in the use of PGE_1 and PGE_2 have precluded their use as therapeutically useful bronchodilator agents, and hopes now rest on the synthesis of analogues without these side effects. Reviews of this work are available [5, pp. 253–285; 8c, pp. 169–224; 11c, pp. 83–102] and some aspects of prostaglandin function in the lung have been reviewed [291].

PGE_1 therapy in peripheral vascular disease. Carlson and Eriksson [292] reported in 1973 that femoral arterial infusions of very low doses of PGE_1 in patients with severe ischaemic peripheral vascular disease was effective in alleviating rest pain and gangrene. More recently Carlson and Olsson have shown that low intravenous doses are also effective [293] and also femoral arterial infusions in patients with verified arterial obstructions produce a dose-related increase in blood flow through the calf of the infused leg [294]. These results suggest that PGE_1 infusion will soon be available for the non-surgical treatment of such diseases.

SYNTHETIC PROSTAGLANDIN ANALOGUES

No prostaglandin analogues have been marketed yet for the treatment of any disease, despite the fact that the first report of the clinical administration of a prostaglandin analogue was reported at a scientific meeting in 1972 [12, pp. 255–264]. Another surprising fact is that despite the generally held belief by workers in this area that numerous prostaglandin analogues have been subjected by tolerance and efficacy trials in volunteers and limited efficacy studies in patients, the great bulk of the published clinical data relates to Upjohn's 15- and 16-methyl substituted PGE_2 and $PGF_{2\alpha}$ analogues. Furthermore, these studies are largely restricted to clinical applications in obstetrics and gynaecology and in the treatment of peptic ulcer disease.

Obstetrics and gynaecology. A summary of the current status of the obstetric and gynaecological use of both natural prostaglandins and their analogues can be found in the published proceedings of the First Inter-Congress of the Asian Federation of Obstetrics and Gynaecology which was held in April, 1976 [14]. The various reviews provide a wide range of references, and the large number of world-wide studies provide some indication of the intense clinical interest in the use of prostaglandins for induction of menstruation (early termination of pregnancy), induction of midtrimester abortions, and for cervical dilation [295]. The Upjohn compounds 15(*S*)-15-methyl-PGE_2 and $PGF_{2\alpha}$ and their methyl esters have been used in the majority of these trials. Depending on the route of administration, there have been reports of side effects of varying degrees of severity. In the case of $PGF_{2\alpha}$ analogues, one of the most serious potential side effects is bronchoconstriction, and there has been a report that several patients receiving 15(*S*)-15-methyl-$PGF_{2\alpha}$ for induction of second trimester abortion have experienced breathlessness [296]. There have been numerous clinical studies on the use of silastic vaginal devices containing 15(*S*)-15-methyl-$PGF_{2\alpha}$ methyl ester, and the proceedings of a meeting on this subject have been published [297]. These 15-methyl compounds seem to be superceding an earlier Upjohn compound, 16,16-dimethyl-PGE_2. Karim had reported that oral administration of this analogue was abortifacient in women [298] although there was a relatively high incidence of gastrointestinal side effects. This compound has been administered vaginally for induction of second trimester abortion [299] and for pre-operative cervical dilation [300]. Interestingly, one study [301] has shown that the *p*-benzaldehyde semicarbazone of 16,16-dimethyl PGE_2 administered as vaginal pessaries to late first and second trimester patients was an effective abortifacient with only mild side effects.

Wiqvist, Martin, Bygdeman and Green [302] have compared the abortifacient doses (i.v.) of a number of prostaglandin analogues in patients in the second trimester of gestation (13th–14th week). The methyl and ethyl esters of $PGF_{2\alpha}$

were 5 times more potent than $PGF_{2\alpha}$ whereas 15-methyl $PGF_{2\alpha}$ and its methyl ester were equipotent, both being ten times more potent than $PGF_{2\alpha}$. Another chemical series, 17-phenyl-PGE_2 (145a) and 17-phenyl-$PGF_{2\alpha}$ were also shown to have lower activities than the 15-methyl prostaglandins. Their most surprising findings were that intravenous infusions of 16,16-dimethyl-PGE_2 (145c) were 400 times more potent than $PGF_{2\alpha}$ and 20 times more potent than 15-methyl-$PGF_{2\alpha}$ in inducing abortion. The great potency of this dimethyl analogue led these authors to suggest that it may have great potential for clinical application as an easily administered vaginal abortifacient. This prediction has to some extent been confirmed by the report that a close analogue, 16,16-dimethyl-trans-Δ^2-PGE_1 methyl ester (Ono 802) [12, pp. 55–66] is a potent abortifacient with few side effects. Finally, in a very recent publication, a new Schering compound 16-phenoxy-17,18,19,20-tetranor-PGE_2 methyl sulphonylamide (SH 286) has been reported to be active in inducing menstruation in patients with a delay in menstruation of 7–14 days [303]. This appears to be better than ICI 81008 (*Figure 8.7*) which has been studied for the same indication [304,305].

Anti-secretory and anti-ulcer prostaglandins. The first prostaglandin found to be orally active in inhibiting basal and pentagastrin-induced gastric acid secretion was 15(*R*)-15-methyl-PGE_2 methyl ester [12, pp. 255–264; 306,307] although the compound was not active intravenously. No side effects were reported, and in a subsequent paper it was shown that an oral anti-secretory dose in pregnant patients at term produced no signs of excessive uterine stimulation [308]. In a comparative study of this analogue with its 15(*S*)-epimer [309], it was shown that the 15(*S*)-epimer was active but was limited by its known uterine stimulant properties in women and by an observed incidence of nausea in the healthy male volunteers used in the study. In follow-up trials in ulcer patients, 15(*R*)-15-methyl-PGE_2 methyl ester was shown to relieve the ulcer pain [310] and to promote the secretion of mucous from the gastric wall as confirmed by endoscopy [311]. There have been more recent reports claiming that this analogue is highly effective in promoting the healing of gastric ulcers in Chinese subjects [312,313], and can inhibit stimulated gastric acid secretions in duodenal ulcer patients [314].

There have been no reports to date demonstrating an anti-ulcer effect in similar patients. One of the significant factors in the activity of this analogue is that the unnatural configuration at the 15-position undergoes epimerisation in gastric juices or in dilute HCl solution to a 1 : 1 mixture of both the 15(*R*) and 15(*S*) compounds [315]. Comparable efforts to epimerise 15(*R*)-15-methyl-PGE_2 methyl ester resulted in only a trace formation of the 15(*S*) compound.

Another Upjohn analogue, 16,16-dimethyl-PGE_2 methyl ester has also been shown to be anti-secretory after oral administration to healthy volunteers [316,317]. Similar studies in patients with duodenal ulcers indicated that much

higher doses were required to produce an anti-secretory effect in these subjects compared with normal healthy controls [318] and at these higher doses a number of side effects were encountered [318,319].

Three prostaglandin analogues, 15(*S*)-15-methyl-PGE_2 and the free acid and methyl ester of 16,16-dimethyl-PGE_2 have been administered via nasogastric tubes directly to the intestine [320,321] and all three compounds had negligible effects at doses which were anti-secretory when administered orally. These results provide additional evidence for a local action of prostaglandin analogues in the stomach. There have also been reports of the comparative activity of these orally administered analogues in healthy volunteers [322,323].

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ADDENDUM

Since this review was completed there has been a rapid increase in the number of papers on prostacyclin synthesis and chemistry [324–337] and stable sulphur [334–336] and nitrogen [337] analogues have been prepared. Other recent chemical papers of note include a chiral synthesis of thromboxane B_2 intermediates [338], synthesis of the methyl ester [339] and an endosulphide analogue [340] of PGH_2 and the synthesis of 15-deoxy-9,11-(epoxyimino)prostaglandins, the latter being potent thromboxane synthetase inhibitors [341].

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Index

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