# PROGRESS IN MEDICINAL CHEMISTRY 13

G. P. ELLIS
G. B. WEST
EDITORS

# Progress in Medicinal Chemistry 13

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# Preface

There are five reviews in this volume and a large part of the book is devoted to clinical enzymology. This is extensively reviewed by Dr D. M. Goldberg (Chapter 1) who considers in detail the clinical applications of enzyme determinations particularly in the field of phosphatases, peptidases, aminotransferases and phosphokinases. This important field of medical science is one which has much advanced in recent years and diagnosis of disease can now often be indicated by abnormal levels of serum enzymes.

The second review by Dr P. F. L. Boreham and Dr I. G. Wright covers pharmacologically active substances released in parasitic infections. Much fresh information in this field has been collected in recent years and the chapter indicates clearly where more detailed work is required.

When a 1,2,3-triazine ring is fused to a benzenoid or aromatic heterocyclic ring, the resulting compounds have been found to exhibit a wide variety of biological activity. Such compounds potentially behave as diazonium compounds and as shown by Dr M. F. G. Stevens (Chapter 3), are thus capable of reacting with a variety of biological materials.

In Chapter 4, Drs Russell and Hopwood review the use of glutaraldehyde as a sterilising agent and as a tissue fixative in microscopy. This interesting compound is now being used extensively in both the chemical and biological fields.

C-Nucleosides are fewer and less well known than their N-counterparts, but they also have some interesting pharmacological and antimicrobial properties; these are surveyed by Drs Daves and Cheng (Chapter 5).

Finally, we wish to thank our authors for their reviews, the owners of copyright of diagrams for permission to reproduce, and the publishers for their co-operation.

*November 1975*

G. P. Ellis
G. B. West

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# 1 Clinical Enzymology

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## INTRODUCTION

In this Chapter, a comprehensive coverage of all aspects of clinical enzymology is not possible, but a sufficiently wide choice of topics has been made to give the reader, it is hoped, the flavour of the subject and a concept of its scope. This choice necessarily reflects in some measure the special interests of the author. After the introductory section which deals with some general technical and biological items, several important enzymes are considered individually and in detail, and attention is given to analytical and clinical facets as well as to molecular forms of the enzyme, where appropriate. The estimation of groups of enzymes in specific clinical situations is then discussed.

Fundamental aspects of enzymology have been lucidly described in the classic text by Dixon and Webb [1]. More extensive and up-to-date information is presented in the celebrated treatise, The Enzymes, now in its third edition [2]. A number of books and monographs on clinical enzymology have been published during the past 12 years [3–7] and they will give the reader a historical perspective. Several books present accounts of various aspects of isoenzymes [8–10] and they should be consulted by those seeking more specialised information.

## INSTRUMENTS AND TECHNIQUES

The first technical innovation that grew out of the special requirements of clinical chemistry was the development of the AutoAnalyser. A flood of papers appeared describing the adaptation of manual enzyme assays to the AutoAnalyser, and these have been catalogued by Roodyn [11]. In essence, these assays were colorimetric, although a UV-absorptiometer and a fluorimeter have occasionally been incorporated for measurement of the final product. This timely advance enabled clinical laboratories to keep pace with the increasing demands being made upon them–in enzyme determinations no less than in any other sub-speciality of clinical chemistry. Moreover, the sequential flow system inherent in the design of the AutoAnalyzer brought improved precision–as much to enzyme assays as to other assays–compared with the manual techniques upon which the methods were founded. Accuracy, being based upon within-run calibration curves, became superior to that obtainable with manual techniques, and sample-interaction or 'carry-over' can always be contained within acceptable limits by suitable design of the reagent manifold and choice of an appropriate 'sample-to-wash ratio'.

The position of colorimetric two-point assays was consolidated around the beginning of this decade with the development of high-capacity multiple analysers capable of performing up to 20 simultaneous tests on each serum sample [12]. Some, however, utilise a device introduced by Trayser and Seligson [13] in which, for each sample, the reaction is initiated twice, the interval between initiation being constant and carefully controlled; the final product is then measured simultaneously in both, so that the difference corresponds to product generated over the fixed time interval. This so-called 'kinetic method' emphasized the desire which became manifest among clinical enzymologists to base determination of enzyme activity upon the continuously-observed rate of reaction rather than upon fixed-time measurements. The advantages and limitations of both approaches have been summarised by Moss [14]. Another stimulus to the use of rate-measurements was the unsatisfactory experience with commercially-produced calibration sera with assigned enzyme values needed for parameter-setting instruments.

Spectrophotometric methods for measuring enzyme activity have been known since the work of Warburg [15], and his 'optical test' involving the interconversion of oxidised and reduced pyridine nucleotides which can be conveniently monitored by absorbance or fluorescence measurements has been expanded to embrace a very wide range of clinically-important enzymes–either directly or through coupled enzyme reactions [16]. A similar service was performed by Kalckar for enzymes of purine metabolism [17] although these are of lesser clinical interest, and the wavelengths required to monitor the reactions are more prone to interference. A decisive advance was the introduction of colourless artificial substrates liberating the yellow *p*-nitrophenate or *p*-nitroaniline on hydrolysis. This principle was first applied to determination of the phosphatases [18], and wide range of substrates for disaccharidases, peptidases, esterases and other hydrolytic enzymes have now become available commercially. From 1960, thermostatted cell-housing blocks and many laboratories have devised procedures to suit their own requirements and simplify the work-load [19].

A few years ago, instruments became available which were capable of automatically transferring, incubating, and initiating tubes or cuvets for enzyme reactions, monitoring the absorbance of the contents, and presenting the rate of reaction on a strip chart recorder or as a digital output. Such 'automatic enzyme analysers' resulted from the growing demands for routine kinetic spectrophotometric enzyme analyses in hospital laboratories and their reception by the profession was prompt and enthusiastic. The first successful instrument of this type was the 8600

Reaction Rate Analyzer (LKB Produkter, Bromma, Sweden), which continues to be the most widely used automatic enzyme analyser in the United Kingdom [20, 21]. Initially limited to output on a strip chart recorder, it can now operate with input to a range of programmable calculators whereby data can be listed as units of enzyme activity [22]. The Gilford 3400 automatic enzyme analyser, unlike the LKB 8600, is a modular instrument which offers a range of temperatures and a wide selection of wavelengths. Whereas to hold serum and reagents the LKB 8600 utilises disposable optical containers which are moved into and out of the light-path, the Gilford 3400 aspirates this mixture into a micro-flow cell and prints the absorbance differences over a series of stepped time-intervals as such or as units of enzyme activity [23].

A third instrument varying the principles of the previous two is represented by the AKES Automatic Enzyme Analyser (Vitatron, Fisons Scientific Apparatus). An assessment of its performance has been published [24]. It incorporates both a sophisticated dispenser unit for automatically adding and mixing serum with the required reagents and a calculator programmed to accept input from the absorptiometer unit and to limit the monitoring period of each assay to what is strictly necessary to obtain a level of precision pre-determined by the operator. An ingenious multi-point sequential flow procedure has recently been introduced where, instead of passing once only through a colorimeter flow cell, the reaction solution passes through three flow cells in tandem and separated by a sufficient time interval to allow a representative portion of the rate curve to be analysed [25].

The 'parallel fast analyser' [26] relies initially on the loading of transfer disks with serum and reagents, a task which can be carried out by automatic dispensers. The disk is then placed in a centrifugal field, which effects mixing of the reaction constituents and propels the mixture into optical cuvets cut out of the periphery of the transfer disk which rotates through a light beam, the signal of which is fed through a photo-sensitive cell to a dedicated computer. Transfer disks with 15–30 cuvet spaces are available with various commercial models and the computer can simultaneously monitor the rate of the reaction in each cell, store the information, and convert it to the appropriate units, either on the basis of a calibration curve constructed from standards included with patient samples on the same transfer disk or using a factor derived from the molar absorption coefficient as in the case of pyridine nucloetide-linked reactions [27–31]. The fact that the signal is continuously monitored by a computer makes it a powerful tool in the application of enzyme kinetics

(as opposed to 'kinetic' enzyme assays) to the problem of characterising and identifying enzymes and iso-enzymes [32]. Other applications to iso-enzyme analysis have also been described [33]. Many other technical developments are on the way [34, 35].

The reagents required to perform enzyme determinations are, in general, more costly and less stable than those used for other routine chemical assays. Although the idea of packaged reagent kits is not exclusive to enzyme assays, such kits are used on a larger scale for enzyme assays than for any other type of assay. A recent survey in Great Britain [36] showed that enzymes were involved in the 6 commonest assays performed with the aid of commercial kits.

The process of lyophilisation, especially as applied to mixtures of reagents, can lead to subtle changes in the products which are poorly understood and not reproducible, but tend to affect their suitability for certain types of enzyme procedures. A well-known problem is the presence in certain batches of NADH of a derivative (as yet uncharacterised) inhibiting lactate dehydrogenase and certain other dehydrogenases [37–39], notably glutamate and malate dehydrogenase [40]. The impact of such impurities upon laboratory quality control can be devastating [41] and recommendations for the detection of inhibitors in NADH have been published [42]. Some commercial samples of NAD also contain a contaminant which inhibits lactate dehydrogenase [43, 44].

A number of bodies have published recommendations for standard reference methods as applied to the commonest and most important serum enzyme assays, notably the national organisations concerned with regulating the practice of clinical chemistry in Germany [45], the United Kingdom [46–48] and Scandinavia [49]. Remarkably, the methods recommended show so little agreement that the uncommitted observer is left in a state of bewilderment and is entitled to question the value of this sort of endeavour. Even an enthusiastic exponent of standardised enzyme assays such as H. U. Bergmeyer [50, 51], has recently proposed an entirely new series of assays for aminotransferase activity [52].

The problem of trace contamination is a much greater hazard of enzyme assays than of most non-enzymatic assays. For example, heavy metals in minute concentration and traces of reaction products or of substrates for auxiliary enzymes in linked reactions may cause serious interference. For these reasons, techniques of quality control are more essential for enzyme assays than for non-enzymatic methods. The lack of suitable quality control or reference enzyme preparations is a major handicap. The specifications required of such preparations have been

formulated [53] but few such preparations have yet been produced, although an aspartate aminotransferase purified from human erythrocytes is reported to meet these requirements [54]. Moss [55] has presented a lucid account of the problems inherent in the quality control of enzyme assays and has made a strong case for the production of immobilised enzymes suitable for this purpose. The challenge has not yet been taken up by industry although immobilised enzymes for many other analytical and preparative procedures are available [56]. Commercial quality control sera, whether in liquid or lyophilised form, are unreliable as reference materials for enzyme assays [57]. Enzymes, in general, are not very stable in solution, and lyophilisation can cause subtle changes in the state of hydration and aggregation of enzymes. A troublesome example of this phenomenon has been well documented for alkaline phosphatase [58–60]. On reconstitution, a steady but not very reproducible increase in activity occurs, followed by a gradual decrease–both processes being strongly influenced by the temperature prevailing during reconstitution and storage. Commercial sera used to calibrate certain enzyme assays and the corresponding enzymes of human serum [61] also show differences in substrate specificity.

## ELEVATED SERUM ENZYME ACTIVITIES

The activity of an enzyme in serum represents a balance between its rate of liberation into the extracellular space and its rate of clearance or uptake from the extracellular space. In health, demographic factors such as age, sex, and body weight also affect this balance in individual subjects [62]. Since the gradient between cells and serum is $>10^3$ for most enzymes (*Table 1.1*), the cell membrane performs a crucial function in retaining enzymes within the cell. A continuous supply of energy, probably in the form of ATP, is necessary to maintain the cell membrane in a conformation optimal for enzyme retention [4]. It follows that absolute anoxia occurring in pathological states such as severe cardiac or respiratory disease may cause significant escape of enzymes from cells, even though no structural damage is evident. Some of the highest serum enzyme activities in the author's experience have occurred in patients with *status asthmaticus*, a largely functional though potentially fatal condition in which anatomical damage to lung and other body tissues is almost non-existent. Even relative hypoxia such as that occurring during severe exercise may cause transient escape of enzymes from skeletal muscle, with consequent elevation of serum activities [63].

Table 1.1. CONCENTRATION GRADIENT BETWEEN LIVER AND SERUM FOR VARIOUS ENZYMES [4]

| *Enzyme* | *Gradient* | *Enzyme* | *Gradient* |
|---|---|---|---|
| Aspartate transaminase | 11600 | Lactate dehydrogenase | 3000 |
| Alanine transaminase | 10000 | Isocitrate dehydrogenase | 16000 |
| Malate dehydrogenase | 4000 | Enolase | 4700 |
| Glutathione reductase | 43000 | Aldolase | 2700 |
| Glutamate dehydrogenase | 7500 | Hexokinase | 14000 |
| Sorbitol dehydrogenase | 48000 | Pyruvate kinase | 700 |

Liberation of enzymes under provocation by phospholipase A or a high potassium ion concentration in the medium, can be prevented by adding ATP or ATP-generating compounds [64]; in the absence of added ATP, the rate of escape of intracellular enzymes is strictly related to the rate of depletion of intracellular ATP. Studies utilising the isolated perfused rat liver showed that anoxia, starvation, or hypoglycaemia led to increased liberation of intracellular enzymes into the perfusate [65]. A striking feature of this work was the demonstration that adverse perfusion conditions were reflected in increased enzyme liberation even when other indices of functional integrity–biliary flow rate, and intracellular ratios of pyruvate/lactate and ATP/ADP–were unaffected.

Drugs and poisons are frequently responsible for raised serum enzyme activities, and presumably may act as inhibitors of energy-yielding processes within cells. Recent evidence, however, suggests that some, notably chlorpromazine and promethazine, act directly upon the cell membrane to stimulate enzyme release [66]. Bacterial toxins also probably act bifunctionally–as metabolic poisons within the cell and externally upon the cell membrane–and generalised septicaemia can cause quite massive increases in serum enzyme activities. Mention should be made of a recent finding that the decay curves for enzymes of normal canine myocardium incubated in vitro are much steeper than those for infarcted myocardium from the same animal [67]. Although the fastest decay in the infarcted muscle may have occurred after injury and before the animals were sacrificed (the interval was 24 hours), this may not be the complete explanation, and careful extrapolation from the in vitro to the in vivo situation is necessary.

Predominance of catabolic over anabolic processes, as a result of cell damage, leads to an accumulation of small molecules (amino acids and sugars) at the expense of large molecules (proteins and polysaccharides).

This in turn increases the intracellular osmotic pressure, an influx of water takes place, the cell swells, the membrane becomes stretched, and pores permitting exchange between the intra- and extra-cellular environments become enlarged and allow larger molecules such as enzymes to escape at a greatly increased rate.

These mechanisms are relatively non-specific, and they serve to explain why serum enzyme elevations are non-specific with regard to the tissue involved and also to the type of injury concerned. The fact that serum enzyme elevations may simply imply transient functional impairment of the cell from which they originate–even although this impairment is consequent upon a serious generalised pathological process–has stimulated the search for indices that might be more clearly related to actual cell necrosis. Such indices are, in fact, available. Those enzymes released from cells during anoxia or as a result of changes in membrane permeability are mainly those present in the cytosol. During cell necrosis, mitochondrial enzymes are liberated. The presence of mitochondrial enzymes in serum is thus evidence for a more severe form of cell injury than that which is required to elevate the serum levels of soluble enzymes. Glutamate dehydrogenase is one such enzyme [68, 69], and aspartate aminotransferase exists in a mitochondrial form which is electrophoretically and chromatographically distinct from the soluble form of this enzyme [70, 71]; its detection is advantageous in assessing the extent of tissue injury and the prognosis for the patient [72, 73]. However experimental studies in the rat after carbon tetrachloride administration showed that whereas the appearance in serum of enzymes from subcellular organelles is qualitative evidence of injury to these organelles, the extent and the timing of the elevation does not necessarily correlate or coincide with the time and severity of the intracellular injury [74].

Other mechanisms lie behind elevation of microsomal or membrane-associated enzymes found in serum. Lipoperoxidation is one such factor [75] and hepatic enzyme induction is another [76]. So far as the liver and biliary tree are concerned, obstruction to the latter causes an increased synthesis of alkaline phosphatase in the liver which, in turn, is reflected in increased serum alkaline phosphatase activity [77]. Evidence from animal experiments suggests that for many enzymes manifesting raised serum levels in the course of bile duct obstruction, bile salts play a crucial role in promoting their liberation from damaged hepatic cells and organelles [78].

The other variable in determining the level of activity of an enzyme in the serum is its rate of clearance [73, 79]. A measure of clearance can be obtained by determining the half-life of the enzyme in clinical subjects,

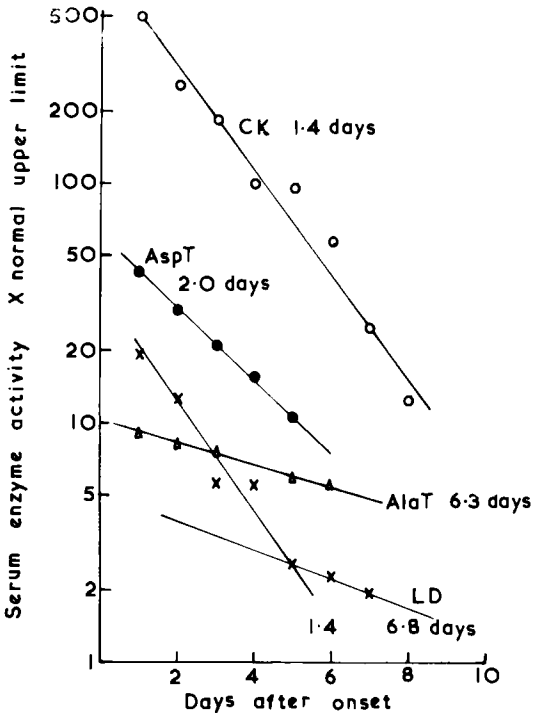


*Figure 1.1. Serum enzyme activities in a patient with idiopathic myoglobinuria, showing the biological half-life for each enzyme [73]. The abbreviations are as follows: CK (creatine phosphokinase); AspT (aspartate aminotransferase); AlaT (alanine aminotransferase); LD (lactate dehydrogenase)–the fast half-life is that of $LD_5$ and the slow half-life that of $LD_1$*

preferably under situations where termination of enzyme release is demarcated, although few such situations exist clinically (*Figure 1.1*). However, results obtained after infusion of enzymes to human subjects suggest that the clearance mechanisms are multiple, and far more complex than can be described by a single half-life measurement (*Figure 1.2*). Experiments involving infusion of ornithine transcarbamoylase in the dog suggest that the process of enzyme elimination has an upper limiting capacity, since the duration of the linear part of the clearance curve was dose-dependent [80]. It is also established that different mechanisms operate for the clearance of different enzymes. Thus, amylase and lipase are cleared in the urine and alkaline phosphatase is excreted in the bile. Neither of these routes contributes significantly to the clearance of lactate or isocitrate dehydrogenase, since the rate is

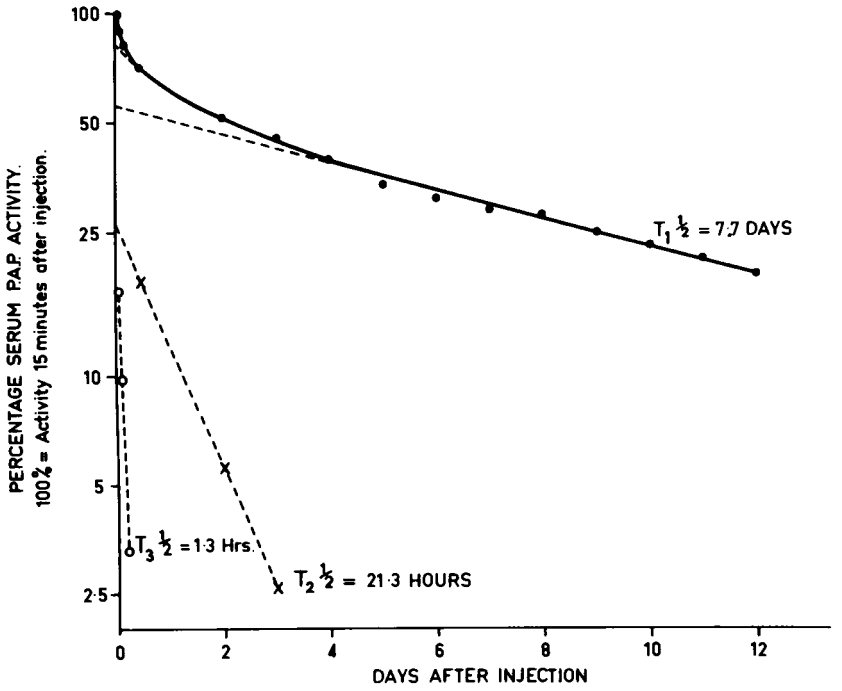


*Figure 1.2. Disappearance of injected placental alkaline phosphatase (P.A.P.) from circulation of a normal 25-year-old male [73]. A solution (30 ml) with activity of 1600 KA units per 100 ml was injected 15 min before zero time. At least three disappearance rates are seen in the early phases. The slow-phase disappearance rate ($T\frac{1}{2}$ = 7.7 days) is similar to that seen when 50 times this initial activity (15.5 King–Armstrong Units per 100 ml) was achieved at zero time by injecting larger amounts of P.A.P.*

unaffected in animals subjected to hepatectomy and nephrectomy [81]. In the dog, the small intestine accounts for 3% and 18% of the clearance of alanine and aspartate aminotransferases respectively [82]. In this animal, the reticulo-endothelial system was shown to be actively concerned in the clearance of soluble aspartate aminotransferase and lactate dehydrogenase but not of alanine aminotransferase, while its role in clearance of mitochondrial aspartate aminotransferase was doubtful [82–86]. Studies in the rat showed that cationic lactate dehydrogenase ($LD_1$) was not cleared by the reticulo-endothelial system (RES), in contrast to anionic lactate dehydrogenase which was actively cleared by this system to such an extent that under conditions of RES-blockade, the clearance rates for both forms of the enzyme were identical [87].

Other removal mechanisms are thought to concern non-biological decay of the type occurring when sera are allowed to stand without refrigeration; gradual degradation by proteolytic enzymes in the serum; or combination with specific inhibitors or antibodies. As evidence for the first two mechanisms, the finding that the $K_M$ of malate dehydrogenase increased progressively in successive serum samples from a patient with myocardial infarction, indicating gradual reduction of substrate affinity, is sometimes cited [88]. However, the $K_M$ for isocitrate of serum isocitrate dehydrogenase of patients with acute hepatitis is similar to that of the human liver enzyme [89], whereas the $K_M$ of serum adenosine deaminase in such patients is much higher than that of the liver enzyme [90]. If increasing $K_M$ were truly related to enzyme degradation, the clearance or decay of adenosine deaminase should be much faster than that of isocitrate dehydrogenase in patients with acute hepatitis. In fact the opposite occurs–the latter is one of the earliest and the former one of the latest enzymes to return to normal values after a clinical attack. Altered $K_M$ values may simply reflect conformational changes following their release from cells rather than degradation of the enzymes in serum.

With use of isotopically-labelled enzymes, information is becoming available that degradation of enzymes within tissues is quantitatively a very important phenomenon. Moreover, tissues vary enormously in the rate at which they degrade enzymes. For example, rat heart muscle degrades $LD_5$ 10-times faster than rat liver and 22-times faster than rat skeletal muscle [91]. Studies in dogs and rats have shown that injected $LD_5$ is taken up by many tissues, especially liver and spleen, in a catalytically active form [92]. Degradation then occurs within the cell. Intravenous administration of creatine phosphokinase to dogs has given results suggesting its uptake by muscle [93].

Therefore, enzymes are taken up by cells and released by cells. The balance of these processes, together with the contribution of other mechanisms such as autodegradation and biliary or renal clearance, determines the serum activities encountered in health and disease. Current knowledge is insufficient to provide an acceptable scientific rationale for all of the serum enzyme alterations seen in the latter, although plausible explanations can be offered for some pathological states. Until our basic understanding of these fundamentals improves, clinical enzymologists will remain obliged to apply facts and information as usefully as they can, suppressing the dissatisfaction that is always aroused by empirical observations, but knowing that immeasurable benefit to patients can nevertheless accrue as a consequence of their skills and efforts.

## ACID PHOSPHATASE (EC 3.1.3.2.; AcPase; orthophosphoric monoester phosphohydrolase)

Estimation of acid phosphatase, one of the first enzymes to gain prominence in clinical diagnosis, has become established as a laboratory procedure in the diagnosis of prostatic cancer [94, 95], and yet there has been much debate in recent years concerning its real utility [96]. Attempts to improve the diagnostic specificity of serum AcPase determinations are being intensified [97].

### METHODS OF ASSAY

These depend on the hydrolysis of a suitable phosphate ester. Rarely, as with β-glycerol phosphate the liberated phosphate is determined [98], and the free alcohol moiety is usually measured. Liberation of phenol [99], *p*-nitrophenol [100], and thymolphthalein [101, 102] from their respective phosphate esters, and of phenolphthalein from its diphosphate [103] or monophosphate [104] can all be measured by simple chemical procedures. Hydrolysis of α-naphthyl phosphate can be followed by a coupling reaction [105] or by spectrofluorimetry [106], and a colorimetric method is available for the measurement of β-naphthyl phosphate hydrolysis [107]. Many of the above methods have been automated by continuous flow techniques [108, 109], and a method using the centrifugal analyser has also been reported [110].

Constant monitoring of AcPase activity is possible with *o*-carboxyphenyl phosphate [111] and with adenosine monophosphates [112, 113]. A colorimetric method based on hydrolysis of 3'AMP has also been presented [114], and use of a constant-current coulometric technique whereby phenol liberated from phenyl phosphate is titrated with bromine has been described [115]. In addition to its role as a phosphomonoesterase, human prostatic AcPase can catalyse the transfer of phosphate radicals from its usual substrates to suitable alcohol acceptors [116].

### ISOENZYMES

#### *Chemical techniques*

The clinical significance attached to prostatic AcPase resulted in a search for chemical methods of identifying tissue-specific enzyme variants. Since the work of Abul-Fadl and King [117], who described the differential effects of various inhibitors (including formaldehyde and L-tartrate)

upon AcPase activity of several organs and cells, it has been common to describe tartrate-labile AcPhase as 'prostatic'. Much subsequent work has shown that tartrate-labile AcPase occurs in other tissues, notably the liver [118–120]. Although $\alpha$-naphthyl phosphate was claimed to be specific for prostatic AcPase [105, 121], it has now been shown not to be so [122], and indeed many years ago it was reported to be hydrolysed by an enzyme present in female urine [123]. Erythrocyte AcPase has a low affinity for $\beta$-glycerophosphate [124], but the range of values using this substrate for serum AcPase activity of normal men and women [95] precludes its specificity for prostatic AcPase. $\beta$-Glycerolphosphate, $\alpha$-naphthyl phosphate and 3'AMP are good substrates for prostatic but poor substrates for erythrocyte AcPase [114].

*Heterogeneity of human prostatic and other AcPases*

Several molecular variants of AcPase have been found in human prostatic and other tissues. Smith and Whitby [125] identified two protein peaks on DEAE-cellulose chromatography and up to 20 bands on starch gel electrophoresis of human prostatic AcPase; they produced evidence that this heterogeneity was due to attachment of varying numbers of sialic acid residues to a single protein. Two distinct forms were separated from hypertrophied prostate by DEAE-cellulose chromatography, and only one form from normal prostate [126]. Two forms with molecular weights of 84000 and 470000 were isolated by gel filtration [127], and further evidence of the heterogeneity of the human prostatic enzyme was provided by the work of Ostrowski and his associates [128, 129]. They reported that micro-heterogeneity is due to variation in neuraminic acid content, and dissociation of an apparently homogeneous form of one of these two enzymes into subunits occurs at extremes of pH values.

Heterogeneity has also been demonstrated using polyacrylamide gel electrophoresis [130, 131] and some prostatic cancers show loss of bands present in extracts of normal prostate [132]. The most comprehensive system of separation has been developed by Yam and his colleagues [97, 133, 134], who have, using polyacrylamide gels, identified 7 zones of activity. Two of these, zones 2 and 4, were in prostate, although zone 2 was also present in white blood cells and pancreas, and zone 4 was in at least 6 tissues besides prostate. Five of the 7 zones were inhibited by L-tartrate. Chemical and electrophoretic methods have so far not provided a satisfactory answer to the organ-identification of AcPase isoenzymes, although recently developed immunochemical techniques may yield fuller results [135, 136].

## CLINICAL UTILITY

### *Prostatic cancer*

The value of serum AcPase activity as an aid in the diagnosis of metastatic carcinoma of the prostate was first established by Gutman and his colleagues [94, 137, 138]. The conclusions of these authors that raised values occur in about half the cases where metastases are to soft tissues only and are uncommon when the tumour remains within the prostatic capsule, have been borne out by most authors who have subsequently reported their experience of large series of cases [122, 139–142].

The usefulness of AcPase estimations, however, has been questioned by Marshall and Amador [143] who made the point that no single case in their series was diagnosed on the basis of serum AcPase activity. The older view concerning the value of serum AcPase measurements in following the progress of the disease, as summarised by Woodard [95], has also been challenged [144, 145]. Consequently there is confusion and suspicion among many clinicians at the present time over the role of this enzyme in the diagnosis of cancer.

Bodansky and Bodansky [146] reported raised serum AcPase activity in 81% of 349 cases with bone metastases and in 24% of 218 cases without bone metastases, and a more recent review [147] supports this incidence. Furthermore, in *Table 1.2*, the incidence of abnormal values obtained in a consecutive series of 46 untreated cases diagnosed in the author's laboratory [148] is compared with some representative reports drawn from

Table 1.2. PERCENTAGE INCIDENCE OF ACID PHOSPHATASE ELEVATION IN PROSTATIC CANCER [148]

| *Reference* | *Sullivan* et al. *[148a]* | *Herbert* *[148b]* | *Woodard* *[148c]* | *Marshall and Amador* *[143]* | *Murphy* et al. *[141]* | *Goldberg and Ellis* *[148]* |
|---|---|---|---|---|---|---|
| Total number of cases | 200 | 82 | 178 | 107 | 185 | 46 |
| Substrate | Phenyl phosphate | | β-Glycerophosphate | | | 3'AMP |
| Stages [141] | | | | | | |
| A | 11 | 42 | 25 | 46 | 45 | 25 |
| B | | | | | 36 | 80 |
| C | | | 60 | 56 | 57 | 100 |
| D | 85 | 89 | 74 | 83 | 88 | 100 |
| All stages | 59 | 62 | 65 | 56 | 64 | 83 |

the literature; as far as is possible, all cases have been grouped into the same 4 stages: A, single nodule; B, more widespread but confined to prostate; C, local extension to soft tissues; D, metastases to bone. Claims for the superiority of tartrate-labile AcPase assays as a diagnostic procedure [139, 149] have not been substantiated [141]. In addition, absence of the prostate appears to make no difference to the activity of tartrate-labile AcPase in serum [149, 150].

*Prostatic massage*

By inducing infarction of an enlarged prostate, catheterisation may lead to gross transient elevations of serum AcPase activity [151]. Hock and Tessier [152] reported elevation of the serum AcPase above the initial value in 17 of 20 patients undergoing rectal examination. In three out of 24 patients with benign cystic hypertrophy, significant elevation of serum AcPase activity also occurred after rectal examination, probably as a consequence of rupture of cysts releasing enzyme-rich secretion into the blood [153]. Elevations were also reported under similar circumstances in some patients with prostatic cancer [154], and as a common sequel to handling of the prostate during transvesical enucleation of the gland [155]. However, other authors have been unable to detect any significant change in serum AcPase levels after rectal examination [143, 156].

*Blood diseases and related disorders*

AcPase is present in high concentration in erythrocytes in the form of 3 isoenzymes responsible for 5 genetically controlled electrophoretic patterns accorded the following nomenclature: A, BA, B, CA and CB [157]. The total content of erythrocyte enzyme is increased in megaloblastic anaemia [158] and is decreased in some patients with hereditary deficiency of glucose 6-phosphate dehydrogenase [159, 160]. Leukocytes are also rich in AcPase, the content of this enzyme being increased in chronic granulocytic leukaemia and decreased in chronic lymphocytic leukaemia and the acute leukaemias [161].

Platelets are also rich in AcPase [162]. For this reason, serum has higher AcPase activity than plasma from the same blood sample [163]. Various thromboembolic disorders such as pulmonary embolism and myocardial infarction cause small increments in serum AcPase activity [164, 165]. Raised serum AcPase activity has also been observed in various leukaemias [166, 167] and in multiple myeloma [168, 169]; in the latter disease, the enzyme may originate in increased osteoclastic activity.

*Other diseases*

Marked elevation of serum AcPase occurs in Gaucher's disease [170]. The enzyme in this condition is resistant to L-tartrate and has a different electrophoretic mobility from those of prostatic isoenzymes [131, 168]. A less marked increase in serum AcPase occurs in Niemann–Pick Disease [171]. It has been claimed, using *p*-nitrophenol phosphate as substrate, that elevated levels are frequent in diabetics–more so in those with vascular complications [172], but this was not confirmed in a study of diabetics in ketoacidosis using 3'AMP as substrate, when a much lower incidence of raised values was observed [173].

## ALKALINE PHOSPHATASE (EC 3.1.3.1.; APase; orthophosphoric monoester phosphohydrolase)

This is the term applied to a group of enzymes with relatively low substrate specificity catalysing the hydrolysis of a wide range of phosphate esters at alkaline pH values. Transphosphorylation reactions are also catalysed by these enzymes, the commonest phosphate acceptors being buffer anions containing alcohol groups [174–176]. Additionally, most such enzymes can hydrolyse pyrophosphate, the optimum pH value in this instance being usually lower than for orthophosphate substrates [177, 178]. The ratio of orthophosphatase to pyrophosphatase activity differs for different tissue forms of the enzyme and may assist in characterising the raised APase activity of pathological human sera with respect to the organ of origin [179, 180]. All APase enzymes thus far purified prove to be zinc metalloproteins with a serine residue at the active centre; this is considered to initiate a nucleophilic attack upon the phosphorous atom of the substrate during break-down of the enzyme–substrate complex [181].

The enzymes are located in the brush borders of the proximal convoluted tubule of the kidney [182, 183] and of the small intestinal mucosa [184], as well as in many other membranes and cell surfaces, including the sinusoidal lining of the liver [185]. In human tissues, they sediment with the microsomal fraction after disruption of the cell [186, 187].

The functions of APases are likely to be diverse and to be dependent upon the tissue concerned, and their location within the cell. Those situated within the external surfaces of absorptive cells are likely to play a

role in transport processes. Indeed, inhibition of intestinal APase has been shown to inhibit fat absorption in man [188]. Its role in bone mineralisation has long been suspected, and its activity correlates with the physiological activity and number of osteoblasts in bones under different functional circumstances [189]. Attempts to define this role in terms of an identifiable mechanism have not been successful so far [190].

No group of enzymes has been so intensively investigated or so heavily utilised for diagnostic purposes. APase assays were among the first to find an established role in clinical chemistry, and even today the number of such determinations performed far surpass those for any other enzyme in every laboratory throughout the world. Recognition of the molecular heterogeneity of APases has led to an explosive interest in APase isoenzymes. This ferment of activity has created a voluminous literature which for several years has been increasing at the rate of 1000 new publications annually. Fortunately, a number of good reviews are available [191–193], while the topic of APase isoenzymes has been comprehensively summarised on two occasions by Fishman [194, 195].

## TECHNIQUES OF ASSAY

For many years the commonest technique was based upon use of disodium phenyl phosphate as substrate, the hydrolysed phenol being determined colorimetrically in a reaction with 4-aminoantipyrine and ferricyanide [99, 196]. Simplified and improved procedures based on these principles have been described [197–199], and a standardized form of the method has been recommended [47]. The original application of this technique for the AutoAnalyzer [200] has been improved [201, 202], and extended for use with second generation sequential flow analysers [61].

Because of the prevailing preference for continuous spectrophotometric methods, *p*-nitrophenyl phosphate is being increasingly used in APase methods, the colourless substrate being hydrolysed to yellow *p*-nitrophenate at alkaline pH [203–205]. This substrate has also been used in an AutoAnalyser method [206], and the necessary reagents are conveniently available in the form of a compressed tablet [207].

Another substrate which has the property of permitting self-monitoring of the enzyme reaction is phenolphthalein monophosphate [208–210]. It has been used in methods suitable for both first and second generation AutoAnalyzers [211, 212].

Thymolphthalein monophosphate is a recently introduced substrate which is gaining in popularity [213, 214]. It shares with the previous two

the property of self-monitoring, since the colourless substrate is hydrolysed to thymolphthalein which is blue at alkaline pH, and it can be conveniently used with the AutoAnalyzer [215]. α-Naphthyl disodium orthophosphate permits hydrolysis to be monitored at 340 nm and can be used with the LKB Reaction Rate Analyser [216]. All those substrates mentioned in the previous Section dealing with acid phosphatase can be used for APase determinations.

From the clinical standpoint there are no overwhelming advantages in any one assay technique; all those commonly used seem equally capable of detecting clinically significant elevations in disease states [217]. However, two problems deserve a mention. The first is the variable behaviour of lyophilised sera which show an unpredictable time-dependent and temperature-dependent increase in APase activity after reconstitution [58]. To a lesser extent, the same phenomena may occur with fresh serum [59]. This has been traced to decomposition of a complex between APase and lipoprotein with liberation of a more active low molecular weight component [60]. This causes major difficulties in the use of lyophilised sera for calibration standards and quality control purposes.

The second difficulty lies in the varying substrate specificity of the different APase isoenzymes [218]. This leads to widely varying activities when commercial reference material, normally derived from a single tissue source (e.g. beef kidney, intestinal mucosa, or human placenta) and therefore unrepresentative of human serum, is assayed with different substrates [219]. For example, disodium phenyl phosphate is a good substrate for all isoenzymes of APase, but *p*-nitrophenyl phosphate is a poor substrate for intestinal and placental APase. When the latter is used in the assay, elevated activities due to these two isoenzymes may easily be missed [220]. When automated systems are used and these depend upon commercial reference material for calibration, there seems to be little alternative to disodium phenyl phosphate as substrate [61].

## ISOENZYMES OF ALKALINE PHOSPHATASE

The exact status of APase isoenzymes is in some doubt [221, 222]. Distinct molecular forms of APase are present in human placenta and small intestinal mucosa and appear to be single gene products. On the other hand, the enzymes in bone, liver and kidney, all of which may be products of the same gene locus but subject to post-transcriptional modification within each tissue, show relatively small differences.

*Electrophoretic separation*

Agar gels have been used with conventional electrophoresis [223] and with high voltage electrophoresis [224] and appear to give good resolution of APase isoenzymes.

Because of its simplicity, cellulose acetate is a popular support medium [225]. The substrates used with this medium have included phenolphthalein monophosphate [226]; $\alpha$-naphthol AS-MX phosphate [225, 227]; 2-hydroxy-3-amido-*N*-(2′,4′-dimethylphenyl)naphthalene phosphate, which yields a fluorescent product on hydrolysis [228]; and $\beta$-naphthyl phosphate incorporated into an agar gel overlay [229, 230]. Starch gel electrophoresis is more difficult, but it provides excellent visualisation of the slow-moving band(s) present in sera of patients with biliary obstruction [231–233]. Polyacrylamide gels have been used, and some authors claim separation between liver and bone bands [234, 235], but high molecular weight forms of serum APase found in biliary obstruction do not usually enter the gel. Prior treatment with neuraminidase does not aid resolution in this medium [236], but treatment with heat or urea which inactivate the bone enzyme can be advantageous [237]. Agarose [238], and Sephadex G-200 gel [239] are other media that have been tried. Isoelectric focusing has been used for some analytical investigations of human tissue APases [240, 241]; removal of zinc from the apoenzyme by the carrier ampholytes is a hazard of this technique.

The migration of different APase isoenzymes and the extent to which they can be resolved varies with the support medium and the buffer used during electrophoresis, but as a rough guide Fishman [195] has suggested the following order of anodal migration; in centimetres: liver (4.4–5.0); bone (4.0–6.0); placenta (3.8–4.2); intestine (3.0). Molecular variants of placental APase exist [242, 243], and genetic polymorphism of this isoenzyme has been demonstrated [244]; consequently, variation in the position of the placental band on electrophoresis may occur from sample to sample, even when the same technique of separation is used. Mention has already been made of the difficulty in visualising high molecular weight components of biliary APase with certain media. The main drawback of electrophoresis is imperfect resolution of bone and liver bands especially when both are present in increased amounts [245].

*Differential inhibition and inactivation*

In the course of studying 130 compounds as possible inhibitors of partially-purified organ preparations of APase in the rat, Fishman and

colleagues discovered the stereospecific inhibition of L-phenylalanine upon intestinal APase [246]. Later kinetic studies led to the proposal that this inhibition was homosteric [247]; a detailed mechanism was suggested, according to which the inhibitor did not affect formation or break-down of the enzyme–substrate complex as such but did block the liberation of inorganic phosphate from the enzyme–phosphate intermediate – the final step in the hypothetical reaction sequence [248]. Following the demonstration of the effectiveness of this compound in identifying human intestinal APase in intestinal fluid and lymph [249], it was used on an increasing scale to quantify this APase isoenzyme in human serum [250]. However, placental APase is also inhibited by L-phenylalanine, and the two isoenzymes cannot be distinguished using this inhibitor [251].

Another stereospecific inhibitor, L-homoarginine, has virtually no effect upon intestinal and placental APase but causes extensive inhibition of bone and liver APase [252]. The length of the hydrocarbon chain and the nature of the terminal basic group are important structural features in determining the action of this inhibitor [253]. It, too, is finding a clinical application, but on a more limited scale. L-histidine acts as an inhibitor of the mixed type upon all forms of APase, but imidazole inhibits only bone and liver APase [254]. The inhibition is uncompetitive and has been postulated to involve $Zn^{2+}$ at the active centre; the intestinal and placental isoenzymes are thought to have $Zn^{2+}$ at other sites which binds imidazole and protects the active centre. Urea has two effects on APase: instant inhibition which is reversible on removal or dilution of the urea, and an irreversible time-dependent inactivation [255]. Bone and kidney APase show greatest susceptibility, placental and intestinal APase least, and liver APase intermediate susceptibility to inactivation by urea [256]. Use of 3 molar urea was claimed to give excellent discrimination between APase in the sera from patients with bone and liver disease; a measure of distinction between sera from patients with liver and biliary disease can be obtained but not sufficient to be of real diagnostic value [257].

Heat stability was formulated as a diagnostic procedure for distinguishing APase, through the efforts of Posen and his colleagues who showed that human placental APase survives heating at 70°C for 30 min in the presence of $Mg^{2+}$ [258], and demonstrated a heat-stable APase in the serum of pregnant females. Indeed, this isoenzyme, now established as placental in origin, accounts for the well-known increase in serum APase activity during pregnancy, and at term it disappears from the serum at a rate equivalent to that determined following infusion of placental APase into non-pregnant subjects [259]. Further study showed that bone and

liver APase are distinguished by heating for 15 min at 56°C, the latter retaining 40 ± 11% of its original activity and the former only 12 ± 6% [260]. No serum from a patient with bone disease retained more than 25% and no serum from a patient with liver disease less than 18% of the initial activity. This approach was criticised by Fishman and Ghosh [194] who pointed out that no allowance was made for intestinal APase present in sera. Nevertheless, the usefulness of these procedures has been validated [261], although the conditions of heating have been varied by different authors who sought to distinguish between the bone and liver isoenzymes; 54°C for 6 min [262], 55°C for 16 min [263], 55.5°C for 15 min [264], 56°C for 20 min [265], and 56°C for 10 min [266] are among those that have been advocated. In the determination of placental APase, heating for 60 min at 55°C and assay at pH 10.7 in the presence of 72 mM phenyl phosphate – a higher pH and substrate concentration than for other isoenzymes – has been recommended [267].

*Other techniques*

The ratio of activities with different substrates was first used in the identification of placental APase [268]. This approach was subsequently applied to the characterisation [249] and determination [269] of intestinal APase.

Separation of APase isoenzymes by gel filtration on Sephadex G-200 has also been achieved, sera from patients with hepato-biliary disease having both a 7 S and a 19 S component [270, 271], but the technique is much too time-consuming for routine purposes. Information from gel filtration studies suggests that the APase isoenzymes from human tissues have the following molecular weights: intestine 125000; liver (principal component) 150000; kidney 175000; and bone 185000 [272]. Immunochemical techniques are also of some value. Monospecific antisera have been prepared for placental [273] and intestinal [274, 275] APase, but the separation of liver and bone APase by immunochemical methods has proved to be more difficult. Some authors have found anti-liver APase antisera to cross-react with bone APase [276, 277], but others have claimed no such cross-reaction when the liver APase used to raise the antiserum is sufficiently pure [278, 279]. Using one such antiserum allegedly monospecific for human liver APase, the claim has been made that the raised APase in the serum of patients with Paget's disease of bone is hepatic in origin [279]. This claim is so contrary to reason, and, being unsupported by parallel data such as heat and urea stability and

electrophoretic mobility of the enzyme, so open to doubt, that the validity of some immunochemical procedures must be seriously questioned.

### *Choice of method*

No single approach, far less a single method, has proved consistently reliable for the identification and quantitation of APase isoenzymes. At the very least, a combination of approaches involving both electrophoresis and use of organ-specific inhibitors is required [237, 280]. Some authors find even these inadequate [245].

One of the main problems in this area is that the property of the isoenzymes as found in pathological sera may not be identical with those of the isoenzyme as isolated from its tissue of origin. This has been shown to be the case for the property of heat stability [281]. It has also been claimed that whereas human bile contains an isoenzyme of $\beta$-mobility identical with the main liver isoenzyme and one of $\gamma$-mobility which appeared to be the liver isoenzyme complexed with phosphatidylcholine [282], the latter isoenzyme is found in the serum of patients with biliary obstruction complexed to a lipoprotein [283]. Another relevant finding is that following bile duct ligation in the rat, the APase extracted from the liver shows increased heat stability and increase in the ratio of orthophosphatase to pyrophosphatase activity [284].

An advantage of the differential inhibition approach is that large batches of samples can be processed and quantitative data obtained by utilising automated procedures. Fractionation of serum into liver and intestinal components has been achieved using an autoanalyser system [285]. A similar approach has been used to screen populations for the placental-type isoenzyme (Regan) found in the serum of some cancer patients [286, 287]. The centrifugal fast analyzer has also been used for analytical dissection of APase isoenzymes [33], and so, too, has the LKB Reaction Rate Analyser [288].

## CLINICAL APPLICATIONS

### *Serum activity in health*

In infancy, APase activity is much higher than in adults [289–291]. After puberty, the levels fall to a minimum. Within-individual and day-to-day variations, are small [292]. During early adult life, values in males are higher than in females, but with increasing age values among females

increase markedly and those in males less so [62, 293–295]. The enzyme in infancy is predominantly of the bone type, whereas in adults it is mainly of hepatic type.

Additionally, the serum of certain subjects may contain intestinal APase. It has now been established that such subjects are persons of blood groups O or A who are secretors of ABH red cell antigens and are Lewis positive [296, 297]. In these subjects, ingestion of a meal rich in fat leads to a striking increase in the amount of intestinal APase in the serum [298–300]. While blood group and secretor status determines the presence of intestinal APase in serum, it has no influence on the occurrence of this isoenzyme in chylous effusions [301]; to account for this observation it has been suggested that the rate of elimination rather than the rate of absorption of this isoenzyme is under genetic control. Reference values for the L-phenylalanine-inhibited fraction of human serum APase in relation to age, sex and blood group have been published [302].

*Serum activity in hepatobiliary disease*

As a general rule, it can be stated that patients with biliary obstruction have high or even very high levels, whereas patients with primary cellular disease of the liver have only moderately increased serum APase activity [303–305]. Although this statement is far from being true in every case, it is so in a sufficient number of cases to ensure that determination of serum APase activity is the cardinal laboratory procedure in distinguishing hepatic from post-hepatic jaundice [306]. In making this distinction, account must also be taken of the degree of icterus and the extent to which serum APase and bilirubin alter during the course of the illness. In extra-hepatic obstruction, the two tend to rise and fall in parallel. Patients with acute hepatic necrosis usually show little change in APase even when the bilirubin is rising to a dramatic height, at least in the early stages of icterus. When icterus fails to clear and the patient enters a cholestatic phase, serum APase activity may then rise substantially, but this is usually a fairly late manifestation [307]. Acute hepatic necrosis may evoke more pronounced increase in serum APase activity among infants than does comparable damage in an adult population [308]. Patients with benign recurrent intrahepatic cholestasis tend to have lower serum APase activities than would be expected for the degree of icterus [309], but the opposite pattern may prevail in some patients with drug-induced intrahepatic cholestasis, depending upon the drug and its site of damage within the liver. Portal cirrhosis is generally associated with normal or only

slightly elevated values for serum bilirubin and alkaline phosphatase activity, but primary biliary cirrhosis is characterised by striking increases in APase activity even when the bilirubin is normal [310]. Apart from primary biliary cirrhosis and bone disease, a raised alkaline phosphatase in the presence of a normal serum bilirubin should alert the clinician to the possibility of metastatic carcinoma or lymphoma infiltrating the liver [311–313]. This is one of the most frequent laboratory presentations of such cases. It has been found that raised serum APase may occur in patients with early lymphoma lacking evidence of hepatic spread [314, 315].

Although much controversy formerly raged over this point, it is now established that the APase found in increased amounts in the sera of patients with hepatic or biliary disease originates from the hepatobiliary system and not from the bone [316–318]. Mention has already been made of the differences between biliary and hepatic forms of APase and their altered characteristics in serum, and attention has been drawn to the prevalence of high molecular weight forms of serum APase in biliary obstruction. An explanation for these phenomena has been proposed by Kaplan based on studies carried out in the rat following bile duct ligation and cholera endotoxin administration [319–322]. In this model, the increase in serum APase is dependent upon prior increase in hepatic APase which can be prevented by cycloheximide or actinomycin D and is therefore attributable to de novo synthesis of the enzyme. The two stimuli differ, however, in so far as endotoxin administration causes an increase in hepatic adenyl cyclase and cyclic AMP prior to the increase in hepatic APase whereas these phenomena are not observed after bile duct ligation. It therefore seems that biliary obstruction causes retention of a chemical inducer which acts independently of cyclic AMP. At present, the question whether the two mechanisms lead to synthesis of the same or two different enzyme proteins has not yet been resolved.

An abnormal electrophoretic form of APase not corresponding to a known hepatic isoenzyme had been reported in the serum of patients with hepatic disease [323]. Later studies established this to be the intestinal enzyme [324–326] and showed that it was raised predominantly in cases of portal cirrhosis. Such cirrhotic patients are generally those of blood groups O and A who are positive secretors [327].

*Serum APase activity in bone disease*

The elevation of serum APase activity in patients with disorders of the skeleton has been recognised for 40 years. Healing fractures are sufficient

to cause such elevation, and the higher normal range of serum APase in infancy testifies to the importance of osteoblastic activity as a determinant of the serum APase activity. Primary bone tumours, such as osteogenic sarcomas, and osteoblastic bone secondaries such as those originating from the prostate, can cause very high levels of serum APase. It is insufficiently recognised that osteolytic secondaries are more often than not associated with raised serum APase although the elevation is only slight to moderate in degree [313]. Other well-known causes include hyperparathyroidism [328], Paget's disease of bone [329], and vitamin D deficiency [330]. The latter problem is becoming of increasing importance among school children in the United Kingdom, especially those from immigrant communities; the serum APase activity is useful in selecting children at risk and in monitoring therapy with vitamin D [331, 332].

Another special group of subjects at risk from metabolic bone disease are those who have had a partial gastrectomy, and indeed in such subjects radiological bone changes accompanied by raised serum APase activity are rather common [333]. Yet an electrophoretic investigation of serum APase in subjects with post-gastrectomy malabsorption revealed that 75% of the raised values were due to excess of the intestinal rather than of the bone isoenzyme [334]. Another study on 463 unselected post-gastrectomy subjects revealed that only those with clinical evidence of Paget's disease or liver disease had abnormal APase activity [335]. The role of serum APase activity in the diagnosis of subclinical bone disease after gastrectomy is thus uncertain.

Hereditary hyperphosphatasia has been reported as a distinct genetic entity transmitted as an autosomal recessive. A failure of cortical bone formation leads to severe deformities of the skeleton with liability to fracture after minimal trauma [336, 337]. A pedigree with the above features, but also manifesting mental retardation and neurologic abnormalities, has been reported [338]; the high APase in the serum of affected infants was characterised by these authors as of liver in type, but since no evidence of liver involvement was presented, this conclusion is somewhat difficult to accept unless one postulates that a mutant form of bone APase chemically similar to that of the liver is the molecular basis for the disease.

The converse congenital abnormality–hypophosphatasia–is also associated with retarded bone development and stunting of the skeleton. In addition to the low APase, there is a deficit of this enzyme in bone, liver and kidney–indeed in all tissues examined, apart from the intestinal mucosa which still retains normal APase content [339, 340]. This fact is one of the lines of evidence suggesting a common genetic control

mechanism for all non-placental non-intestinal APases. The increased excretion of ethanolamine phosphate in this condition has been recognised for 20 years [341]. A more recent finding, and one which links APase with metabolism of inorganic pyrophosphate, is that patients with this disease also excrete increased amounts of this compound in their urine [342].

*Serum APase activity in pregnancy and placental function tests*

Using an automated technique, Fishman has defined the range of serum placental APase activity in pregnant women and has shown that it follows an exponential course as a function of gestation time [343, 344]. The lag period before the enzyme can be detected, following conception, averages 6 weeks in healthy women and 10 weeks in diabetic mothers; the blood group also influences the duration of the lag period. While this is the most thorough study of placental APase in pregnancy, its relevance is hard to grasp at the present time in view of the adverse opinions expressed by previous authors concerning the value of placental APase in monitoring foetal well-being. Some workers had noted a steeper-than-usual rise in serum placental APase in patients with pre-eclampsia or other form of placental dysfunction, even weeks before clinical signs of such complications were evident [345–348]. However, subsequent studies demonstrated that complex criteria were needed to evaluate serum placental APase activity, since placental dysfunction can result in abnormally high activity, abnormally low activity, or falling activity within the normal range [346, 349–351]. Indeed this 'normal range' is itself so wide in both the amplitude and the upper limit as term approaches (*Figure 1.3*) that only multiple serial estimations enable timely predictions to be made.

Placental APase is only one of several enzymes in maternal serum which reach their highest values near term. They have been utilised as tests of placental function to determine whether the survival of the foetus is being threatened by incipient degeneration of the placenta; if it is, and when the foetal age is adequate, a premature birth can be induced. Falling values of diamine oxidase activity have been reported to correlate with a greatly increased incidence of foetal death [352, 353], but this statement has been challenged [354].

Leucine aminopeptidase [355, 356] and cystine aminopeptidase [357–360] show similar increases in activity in late pregnancy; the latter hydrolyses oxytocin, but in fact both activities may be due to a single

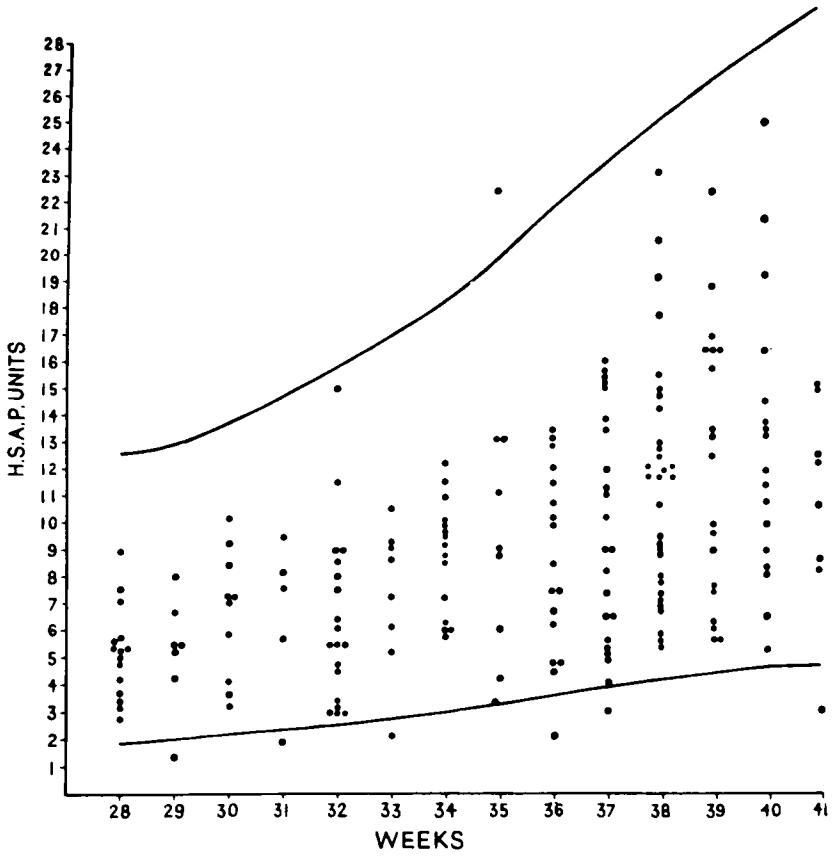


*Figure 1.3. Heat-stable alkaline phosphatase activity of maternal serum in relation to time of gestation [349]. The curves indicate 95% confidence limits. One H.S.A.P. unit is the amount of enzyme liberating 1 mg phenol from 36 mM disodium phenyl phosphate in 15 min at 37°C at pH 10.7 in bicarbonate buffer*

exopeptidase of placental origin. All reports agree that values are high near term in healthy pregnancies; lower values are found in the maternal serum when there is evidence of foetal distress, and very low values where the infant is stillborn. However, the degree of resolution between these groups is not sufficiently clear-cut to allow firm conclusions to be reached on the basis of individual determinations, and serial measurements are required.

*Ectopic tumour APase isoenzymes*

One of the most striking advances in our understanding of the biology of APase isoenzymes has come from recognition of the presence of abnormal molecular variants of APase in the serum of some patients with carcinoma. The first to be described, the Regan isoenzyme, is heat-stable, sensitive to L-phenylalanine, runs on starch gel electrophoresis in the position expected of placental APase, cross-reacts with antiserum to the latter, and is indistinguishable from it [361, 362]. A similar APase, sensitive to L-phenylalanine and resistant to heat, is present in HeLa cells – a strain of cultured cells widely available for experimental purposes but originally derived from human carcinoma. The molecular properties of Regan isoenzyme and placental APase are identical except that the former is 6-fold more sensitive to inhibition by urea [363]. The discovery of this enzyme, a product of the foetal genotype [364], came at a time when other foetal proteins, notably $\alpha$-foetoprotein and carcinoembryonic antigen, were being recognised in the serum of human cancer patients. The concept of de-repression of the genome was advanced to explain this reversion in ability to synthesise a primitive foetal protein not found in normal adult tissues. The incidence of the isoenzyme was reported to be 27 of 590 cases of malignant disease [365], but Fishman [195] now claims an incidence of one in seven when sensitive assay procedures are used. These claims await confirmation, but the implications of Fishman's discovery are exciting and stimulate a search for other aberrant APase isoenzymes in cancer.

Timperley and his colleagues reported the presence in serum and in lung tumour of an APase sensitive to heat and insensitive to L-phenylalanine [366]. The same group has found large amounts of APase with similar properties in other human tumours, including meningiomas, cranio-pharyngiomas and tumours of the exocrine and endocrine pancreas [367–369]. These isoenzymes are different from Regan isoenzyme, but it is not yet clear to what extent they differ from other forms of non-placental non-intestinal APase.

Shortly after the original report on Regan isoenzyme, a variant APase was found in tumour extracts from 8 of 10 patients with hepatocellular liver cancer and only two of 60 livers free of cancer [370]. Subsequently a similar variant was reported in serum, ascitic fluid and tumour tissue of 3 out of 10 hepatoma patients, and a 300-fold purification of the enzyme was achieved [371]. Its properties seem intermediate between those of liver and placenta in that it is moderately sensitive to heat and greatly inhibited by L-phenylalanine; electrophoretically, however, it migrates

ahead of the fastest liver component. Fishman has speculated that it may be a hybrid composed of subunits of both liver APase and the rare 'D' variant placental APase [195]. This latter is sensitive to L-leucine as well as to L-phenylalanine, a property shared by the hepatoma variant.

Yet another variant, called Nagao isoenzyme, was reported in pleural carcinomatosis and cancer of the bile duct [372, 373]. This appears to be an ectopic form of the 'D' variant placental APase, being identical with the usual placental and Regan isoenzymes in heat stability and L-phenylalanine sensitivity, but differing in being inhibited by L-leucine and by 4 mM EDTA. Up to 4 high molecular weight variants of APase have been detected on cellulose acetate electrophoresis and gel filtration; they are completely inhibited by heating at 65°C [374]. One moves ahead of the main liver band and has inhibition properties similar to bile APase; the other 3 have inhibition properties similar to those of intestinal APase. While most variant APase isoenzymes have been described in tumours or the serum of subjects with carcinoma, one rare form found in the serum of 4 out of 19 patients with ulcerative colitis was notable for its exceptionally slow electrophoretic mobility and for the fact that it was heat labile and resistant to L-phenylalanine [375].

*Other aspects of APase*

In general, intestinal APase does not enrich the serum in patients with intestinal disease. An exceptional case of severe steatorrhoea of unknown cause has however been reported in which abnormally high serum APase activity was shown to be due to an excessive amount of intestinal APase [376]. Patients on haemodialysis show increased APase activity and this appears, on the basis of electrophoretic and inhibition studies, to be predominantly intestinal [377, 378]. An increase in thermostable serum APase has been stated to occur in tuberculoid leprosy [379]. This activity was not properly characterised, but the authors seemed to regard it, in the absence of any supporting evidence, as representative of Regan rather than of intestinal isoenzyme. Patients with thyrotoxicosis had a higher mean APase activity than a comparable group of euthyroid or myxoedematous subjects, and levels outside of normal limits were occasionally noted [380]. Sporadic and unexplained elevation of serum APase activity may also be found among diabetic patients [381].

Urinary APase activity is measurable after dialysis of the sample to remove inhibitors. Raised levels measured over 8 hours were found in 12 of 13 patients with renal adenocarcinoma, 6 of whom were asymptomatic [382]. Elevated urinary levels, however, also occur in subjects with renal

parenchymal disease such as acute or subacute glomerulonephritis [383].

Leucocyte APase activity has been frequently utilised as a test for leukaemias, since decreased histochemical activity may be observed in these conditions [384, 385]. Cyclical variations occur in the female during the reproductive years, and activity increases in response to endogenous or exogenous oestrogens [386]. Attempts to purify and characterize the enzyme have been made. It is strongly dependent on zinc for its activity, and very sensitive to inhibition by a wide range of amino acids, especially cysteine and histidine [387].

APase is present in the human duodenal aspirate, and its concentration increases dramatically after administration of secretin and cholecystokinin-pancreozymin [388, 389]. Human pancreatic juice contains negligible amounts of APase and the response to the above hormones is obtained in patients who have had their gall-bladders removed. This, and more direct evidence from characterization of the enzyme, indicates that release of APase from intestinal mucosa is stimulated by these hormones. In the rat, stress or cortisone administration increases intestinal APase activity [390].

## 5′-NUCLEOTIDASE (EC 3.1.3.5.; 5Nase; 5′-ribonucleotide phosphohydrolase)

The main clinical interest in this enzyme is concerned with its utility in the

Table 1.3. PROPERTIES OF VARIOUS 5′-NUCLEOTIDASES [396]

| *Enzyme source* | *Cellular localisation* | *Substrate specificity*[a] | *pH*[b] *optimum* | *Activators* |
|---|---|---|---|---|
| Rat liver | Supernatant | 5′dGMP, GMP, IMP | 6.3 | $Mg^{2+}$ |
| Rat liver | Supernatant | 3′- and 5′-nucleotides and deoxynucleotides | 5.6–6.4 | $Mg^{2+}$ |
| Rat liver | Lysosomes | 2′-, 3′- and 5′-nucleotides, 5′-deoxynucleotides | 3.75–5.5 | None |
| Rat liver | Plasma membrane | UMP, AMP, CMP | 7.5, 9.3 | $Mn^{2+}$, $Mg^{2+}$ |
| Calf intestinal mucosa | Not stated | UMP, CMP, GMP | 6.0–6.5, 8.0 | $Co^{2+}$, $Mn^{2+}$, $Mg^{2}$ |
| Pig intestinal smooth muscle | Plasma membrane | AMP, CMP, IMP | 7.0, 5.5<br>8.5–9.8 | $Mg^{2+}$ |

[a] Where established, the 3 most active substrates are given in the order of activity sustained by them.

[b] Multiple pH optima are listed in the order of activity obtained.

diagnosis of hepatobiliary disease. It has a wide tissue distribution, highest activities being found in endocrine organs and vascular endothelium [391], but liver is not especially notable for its 5Nase content. Evidence from histochemical and electrophoretic studies suggests that it exists as a number of isoenzymes [392, 393]. Until recently, it was widely believed that 5Nase was restricted to the plasma membrane, and its activity was employed to determine the purity of plasma membrane fractions [394], but no less than four distinct enzymes with 5Nase activity have already been purified from rat liver. They differ in their catalytic properties and in the cell locus to which they are confined (*Table 1.3*). A number of reviews provide coverage of the fundamental biochemical aspects of 5Nase [395–398], and no further attention will be devoted here to the molecular heterogeneity of 5Nase enzymes since advances in this field have not been translated into useful clinical procedures.

## TECHNIQUES OF ASSAY

The fundamental reaction in most methods for determining serum 5Nase activity is the following:

$$\text{adenosine 5'-phosphate} \rightarrow \text{adenosine} + P_i \qquad (1)$$

Early methods measured the inorganic phosphate liberated during a fixed incubation time. Since the substrate, adenosine 5'-phosphate (AMP) is also hydrolysed by alkaline phosphatase, attempts were made to compute and deduct this hydrolysis from the total activity by carrying out a parallel determination using a substrate for alkaline phosphatase [399, 400], using the differential inhibition effects of EDTA upon the two enzymes [401], the inhibition by $Ni^{2+}$ of 5Nase [402], and the activation by $Mg^{2+}$ of 5Nase [314]. None of these methods is sufficiently specific to contend with interference by very high alkaline phosphatase activity in the 5Nase assay; furthermore, problems of determining inorganic phosphate, especially the small activities encountered in the normal range, lead to poor precision.

Alternative methods are based upon measurement of adenosine, liberated from AMP according to Equation (1) above, utilising the specific enzyme adenosine deaminase which catalyses the following reaction:

$$\text{adenosine} \rightarrow \text{inosine} + \text{ammonia} \qquad (2)$$

Conversion of adenosine to inosine results in a fall in absorbance at 265 nm, the absorbance maximum for adenosine [17]. This principle forms the basis for methods suitable for measuring 5Nase activity of human

serum [403–405]. Colorimetric methods, in which ammonia liberated according to Equation (2) is measured in a fixed-time assay by means of the phenol-hypochlorite (Berthelot) reaction, have also been developed for human serum 5Nase [406, 407] and applied to automated sequential flow systems [408, 409]. An advantage of all these methods based upon coupling 5Nase activity to that of adenosine deaminase is that the action of alkaline phosphatase upon AMP can be suppressed by including in the medium an excess concentration of an alternative substrate for the latter which will act as a competitive inhibitor of AMP hydrolysis by alkaline phosphatase without affecting the specific hydrolysis of AMP by 5Nase. Both $\beta$-glycerophosphate [403, 404] and disodium phenyl phosphate [410] have been used for this purpose, but there are strong reasons for preferring the former [411, 412].

A third approach consists of coupling the two reactions described in Equations (1) and (2) above with a third reaction, the reductive amination of 2-oxoglutarate catalysed by glutamate dehydrogenase:

$$\text{2-oxoglutarate} + NH_3 + NADH + H^+ \rightleftharpoons \text{L-glutamate} + NAD^+$$

This enables the reaction to be followed by monitoring the decrease in absorbance at 340 nm [413, 414]. An advantage of this procedure is its suitability for automatic enzyme analysers, and one such application has been presented [21, 415].

## CLINICAL APPLICATIONS

### *5Nase and alkaline phosphatase*

A series of reports during the years 1954–1964, ably summarised in the review by Bodansky and Schwartz [395] established the value of serum 5Nase assay in elucidating the source of raised serum alkaline phosphatase activity in anicteric subjects. It was based upon the following argument: serum alkaline phosphatase activity is raised in diseases of the bone and the liver; serum 5Nase activity is raised only in diseases of the liver; therefore when both are raised, the alkaline phosphatase must be of hepatic origin, and when only alkaline phosphatase is raised, the abnormality must signify bone disease. However, 5Nase may be occasionally elevated in diseases other than those of the liver and biliary tree, and alkaline phosphatase isoenzymes exist in tissues other than those of bone

and liver. Statements about the presence of organ-specific isoenzymes in serum in abnormal concentration cannot properly be based upon assay of the activity of a different enzyme, although the technical and interpretative difficulties in separating alkaline phosphatase isoenzymes make this a tempting, albeit unreliable, alternative. However, 5Nase is not the only enzyme which can and has been used for this purpose. Leucine aminopeptidase [416–418] and GGT* [419–421] have also been assayed in attempts to define the cause of raised serum alkaline phosphatase activity, and there is no reason to consider 5Nase as superior to the latter two enzymes in this regard.

*5Nase in hepatobiliary disease*

Many reports testify to the fact that serum 5Nase activity is raised predominantly in disease of the biliary tree, although activities in subjects with primary hepatic parenchymal diseases generally show only modest elevation [395, 397, 398]. Whereas a very high activity is strongly suggestive of biliary tract disease in a jaundiced patient, there is no arbitrary value that can reliably segregate patients with biliary and hepatic diseases [422, 423]. Studies correlating 5Nase activity with clinical progress and with histological features apparent in liver biopsy samples indicate that biliary ductules are the major source of the elevated serum 5Nase and that the assay is a sensitive indicator of diseases affecting them, especially when these induce proliferation or destruction of bile ducts, or inflammatory cell infiltration within the portal tracts [397, 404, 423].

Much discussion and controversy has centred upon the comparative value of 5Nase and alkaline phosphatase in the investigation of patients with hepatobiliary disease. One study suggested that 5Nase was superior [422]. Others indicated a preference for alkaline phosphatase [418, 424–426]. The criteria for selection of sera in the latter group of investigations were such as to bias the comparison in favour of alkaline phosphatase. Three reports are summarised in *Table 1.4.* In the first two, sera were selected on the basis of raised alkaline phosphatase or hyperbilirubinaemia; 5Nase was less sensitive in all categories of hepatobiliary disease. In the third, both enzymes were routinely measured, without prior selection, in all sera submitted with suspected hepatobiliary disease; 5Nase was superior to alkaline phosphatase in detecting parenchymal

* GGT; (γ-glutamyl transpeptidase).

Table 1.4. COMPARATIVE VALUE OF SERUM APase [99] AND 5Nase ACTIVITIES IN HEPATO-BILIARY DISEASE

| | *Magnesium activation [314]* | | *Manual kinetic at 265 nm [404]* | | *Automated kinetic at 340 nm [415]* | |
|---|---|---|---|---|---|---|
| | *Raised APase* | *Raised 5Nase* | *Raised APase* | *Raised 5Nase* | *Raised APase* | *Raised 5Nase* |
| *Extrahepatic obstruction* | 100 (55)[a] | 72.7 | 100 (48) | 91.7 | 94.0 (67) | 95.5 |
| Simple (clinical diagnosis) | 100 (16) | 56.3 | 100 (13) | 76.9 | 90(9) | 90 |
| Simple (operative diagnosis) | 100 (7) | 85.7 | 100 (7) | 100 | 100 (10) | 100 |
| Inflammatory | 100 (10) | 90.0 | 100 (10) | 100 | 77.8 (18) | 83.3 |
| Malignant | 100 (22) | 77.3 | 100 (18) | 94.4 | 100 (30) | 100 |
| *Hepatic disease* | 85.9 (64) | 48.4 | 82.2(76) | 69.7 | 69.4 (98) | 82.7 |
| Viral hepatitis | 77.3 (22) | 36.4 | 83.9 (31) | 67.7 | 83.9 (31) | 83.9 |
| Micronodular cirrhosis (clinical diagnosis) | 83.3 (6) | 33.0 | 100 (6) | 66.7 | 50 (18) | 83.3 |
| Micronodular cirrhosis (histologic diagnosis) | 84.2 (19) | 47.4 | 80 (20) | 60 | 55.6 (36) | 75 |
| Primary biliary cirrhosis | 100 (11) | 81.8 | 100 (10) | 100 | 100 (13) | 100 |
| Miscellaneous | 100 (6) | 66.7 | 100 (9) | 66.7 | — | — |
| *Hepatic secondaries* | 100 (31) | 87.1 | 90.6 (32) | 81.3 | 79.1 (43) | 83.7 |

[a] Open figures are percentages and those in parenthesis are actual number of cases.

liver disease, and marginally superior in detecting disease of the biliary tree and hepatic metastases.

In the neonate and infant, 5Nase has advantages over alkaline phosphatase as the latter is high throughout this period and further increases occur during growth spurts; because the growth rate is frequently altered as a result of disease in infancy and in many primary and secondary diseases or disturbances of bone, these conditions will be reflected in an altered serum alkaline phosphatase activity. These disadvantages do not apply to 5Nase which is lower in serum during infancy than during adult life [291]. Consequently 5Nase is superior to alkaline phosphatase in the frequency and magnitude of the elevations occurring in hepatobiliary disease during childhood and in the specificity of such elevations, as they are rarely encountered in other childhood illnesses [291, 422, 423]. It has been reported that serum 5Nase activity is especially useful in distinguishing biliary atresia from neonatal hepatitis as values were invariably very high in the former and only moderately high in the latter, and the two diseases segregated without overlap [427]. If confirmed, this would be a valuable diagnostic aid since it is extremely difficult to differentiate between these two important causes of persistent jaundice in the newborn period.

*5Nase in cancer*

Raised serum 5Nase activities occur in a high proportion of patients with spread of metastatic tumour to the liver [312, 313, 428]. This can occur before the onset of jaundice. Although most such patients also have raised serum alkaline phosphatase activity, this is not invariable, and 5Nase seems to be the more reliable test. Sequential determination of serum 5Nase activity is also valuable in monitoring progress of the disease [429, 430], since it falls more rapidly than alkaline phosphatase after successful chemo- or radio-therapy, and rises more rapidly during recurrence. A lipid–protein complex with 5Nase activity was found in the serum of two-thirds of 44 patients with hepatic cancer and none of 40 cases with other hepatobiliary diseases [431]. There is no evidence that serum 5Nase activity is useful in the diagnosis of primary cancers before spread to the liver occurs.

*Other diseases*

Raised serum 5Nase was found in association with raised alkaline phosphatase and GGT activities in a high proportion of subjects 4 to 10

days after myocardial infarction [432]. Serum 5Nase was reported to be elevated in 30% of 66 patients with rheumatoid arthritis, and very high levels were obtained in the synovial fluid of 58% of 24 subjects tested [433]. Hepatic enzyme induction may prompt an elevation of serum 5Nase [434], and raised activities have been found in 25% of cases of diabetic ketoacidosis [435].

In the author's experience, unexplained elevations of serum 5Nase activity occur in about 10% of patients suspected of having hepatobiliary disease in whom the diagnosis cannot be confirmed. This diminishes the specificity of the assay, although most such elevations tend to be relatively slight. 5Nase remains one of the most specific enzyme tests for hepatobiliary disease, and levels exceeding twice the upper normal limit are not often encountered in other conditions.

## γ-GLUTAMYL TRANSPEPTIDASE (EC 2.3.2.1.; GGT, D-glutamyltransferase)

This enzyme is distributed in many tissues of the body, highest activity being located in kidney, then pancreas, liver, spleen and placenta [436, 437]. It catalyses a reaction between glutathione (γ-glutamylcysteinylglycine) and all of the common protein amino acids except proline:

$$\gamma\text{-glutamylcysteinylglycine} + \text{amino acid} \rightarrow$$
$$\gamma\text{-glutamyl-amino acid} + \text{cysteinylglycine}$$

Other γ-glutamyl-peptides apart from glutathione may function as substrates for transpeptidation by this enzyme, and synthetic amides of glutamic acid may also serve in this capacity [438]. It plays an important role in the reabsorption of amino acids from the glomerular filtrate within the renal tubule [439], and is present in the endoplasmic reticulum of the hepatocyte where its activity is increased in situations leading to microsomal enzyme induction [440]. The walls of the bile canaliculi are richly endowed with GGT activity, but there appears to be considerable species variation with regard to whether the biliary epithelium or the hepatocyte is the predominant GGT-containing cell of the hepato-biliary system [441]. A claim that a soluble form of the enzyme exists in human tissues [442] must be questioned as it is based on study of autopsy material using crude homogenisation procedures.

## TECHNIQUES OF ASSAY

The assay of GGT activity is dependent upon the availability of synthetic substrates. These are predominantly derivatives of glutamic acid in which a diazotisable radicle or a chromogenic ion is coupled to the γ-carboxyl group. The first to be used were α-(*N*-γ-DL-glutamyl)-aminopropionitrile [443] and *N*-(DL-γ-glutamyl)-aniline [444]. Synthesis of γ-L-glutamyl-α- and -β-naphthylamides extended the range, the former being preferred because of its greater solubility although enhanced activity was obtained with the latter [445]. In the above procedures, the reaction is stopped by protein precipitation and an aliquot of the protein-free filtrate taken for diazotisation employing the Bratton-Marshall reaction. Additionally, two molecules of substrate were used for each transpeptidation step, since one acted as acceptor for the glutamyl group transferred from the other, thus:

γ-L-glutamyl-α-naphthylamide →
γ-L-glutamyl-γ-L-glutamyl-α-naphthylamide +
α-naphthylamine

An important development comprised the use of glycylglycine as acceptor for the γ-L-glutamyl residue [446, 447]. Fast blue B salt was used as coupling agent instead of the more cumbersome Bratton-Marshall reaction, while other workers [448] selected conditions that avoided the need for protein precipitation.

However, the commonest substrate used at the present time is γ-L-glutamyl-*p*-nitroanilide. Transfer of the γ-glutamyl residue to glycylglycine releases *p*-nitroaniline which, at the pH for optimal activity, absorbs strongly at 405–420 nm. This property may be used to monitor the reaction continuously [449] or it may be used to measure the concentration of free *p*-nitroaniline after taking steps to terminate the enzyme reaction [450–452]. An alternative approach involves diazotisation of the *p*-nitroaniline to overcome the problem of jaundiced sera and to yield a more sensitive assay [453]. Automated methods based on this substrate have been devised [454, 455]. A recent modification incorporates glutamate in a concentration of 1 mmol/litre into the reaction mixture, since this enhances activity by 20–100% [456]. Kinetic methods using this substrate are preferable to fixed-point assays since the free *p*-nitroaniline is inhibitory and therefore the reaction should be completed in the shortest possible time [457]. It has been reported that glutathione, phenol tetrabromophthalein sulphonate and L-serine reduce activity with some of the above synthetic substrates [444].

## CLINICAL APPLICATIONS

### *Hepatobiliary disease*

The earliest clinical studies demonstrated that GGT activity of serum was raised in a variety of diseases affecting the liver, biliary tract and pancreas [436, 444, 458]. The enzyme displayed highest activities in those diseases where biliary stasis was the dominant pathological feature and high activities were also observed in association with space-occupying lesions such as hepatic metastases. Subsequent investigators therefore tended to concentrate on these two aspects. A substantial number of reports have been devoted to comparisons of GGT determinations and those of other marker enzymes of biliary stasis such as alkaline phosphatase and leucine aminopeptidase [419, 440, 459–464]. The overwhelming consensus is that GGT determinations run a course parallel to that shown by the latter enzymes, but the relative elevations of GGT are very much greater, conferring superior sensitivity in diagnosis of biliary tract disorders, although diseases of the pancreas are also associated with very high activities. Several investigators have noted that normal GGT activities generally prevail in bone diseases where alkaline phosphatase levels tend to be elevated; the test may therefore be used to determine whether bone or liver is the probable source of raised APase activity [420, 421, 464–467].

There is less uniformity concerning the behaviour of GGT in primary hepatic disease. In an experimental study, carbon tetrachloride administration, in contrast to bile-duct ligation, failed to elevate serum GGT activity [440]. Not surprisingly, therefore, these authors reported that only moderately raised activities–or even normal values–were encountered in viral hepatitis [440, 468]. While few would dispute that values in hepatitis are generally lower than those in biliary stasis, there is too much overlap to enable accurate discrimination between these conditions to be made on the basis of serum GGT assays alone [464–466]. Values tend to be very variable in cirrhosis. Occasionally they may be very high [459, 469, 470] and may even be the only serum enzyme activity to be elevated [471]. At other times they may be normal [440, 466]. In part, this variability may be due to a tendency for serum GGT levels to decline as the condition reaches a terminal phase and hepatic synthesis of GGT becomes impaired [458, 469, 472, 473].

The value of GGT in detecting hepatic metastases is well documented [448, 460, 470, 471, 474]. However, the most authoritative study on this topic led to the conclusion that the test was an embarrassment rather than an aid and would have resulted in serious errors of classification in a

series of over 200 patients with established primary carcinomas if reliance had been placed upon it [475]. It has also been stated that the tendency of serum GGT to be raised in response to cytotoxic drug therapy makes it unreliable in monitoring spread of the malignant disease [455]. An experimental study to determine the mechanism of the raised serum GGT in patients with hepatic metastases revealed increased enzyme in the hepatocytes adjacent to the tumour as well as in the tumour itself; the authors considered that intra-hepatic cholestasis contributed to the elevated levels within the hepatocytes [476].

*Chronic alcoholism and enzyme induction*

Elevated levels of serum GGT activity have been described in this condition and are frequently the only abnormalities encountered [470, 477, 478]. Detailed studies have given rise to a rather confusing picture [479, 480]. Raised values are seen almost as frequently in alcoholics without tangible evidence of liver disease as in those with clear stigmata of hepatic involvement. Moreover, these reports document a high proportion of abnormalities among subjects who are defined as heavy drinkers but not alcoholics; a lesser proportion of abnormalities was seen in moderately heavy drinkers, and even some teetotallers had raised GGT activities.

This situation is partly explicable in terms of the well-known phenomenon of hepatic microsomal enzyme induction which occurs after exposure to alcohol and to certain drugs, especially barbiturates, antidepressants and anticonvulsants [481, 482]. It has now been established that raised serum GGT activities occur in patients to whom these drugs are administered therapeutically, epilepsy and psychiatric illness figuring prominently in this respect [419, 434, 483]. The previously-cited studies have not taken account of the possible role of these drugs which are likely to be prescribed for a proportion of alcoholic subjects. Raised serum GGT activity in a suspected alcoholic may therefore arise as a consequence of alcohol-induced hepatic damage, alcohol-induced microsomal enzyme synthesis in an otherwise healthy liver, or drug therapy administered to the alcoholic subject. A recent report mentioned that GGT was abnormal in alcoholics no more frequently than other tests of liver function [477]. A normal value has never been obtained in any of 60 proven alcoholics tested in the author's laboratory, and activities up to 20-times the normal limit are by no means uncommon in such patients even when all other biochemical tests are normal [484].

The potential use of serum GGT as an index of microsomal enzyme

induction, and hence of drug exposure, is of great interest to psychiatrists who are often faced with the problem of determining whether a patient manifesting a poor response to drug therapy has actually taken the drugs prescribed. The kind of response to drug administration which may be encountered clinically is demonstrated in *Figure 1.4.* Comparison with other indices of microsomal enzyme induction has shown GGT to be at least as reliable and technically much easier to perform [485].

*Myocardial infarction*

It has been shown by many investigators that serum GGT activity increases after myocardial infarction; since the increase persists for about

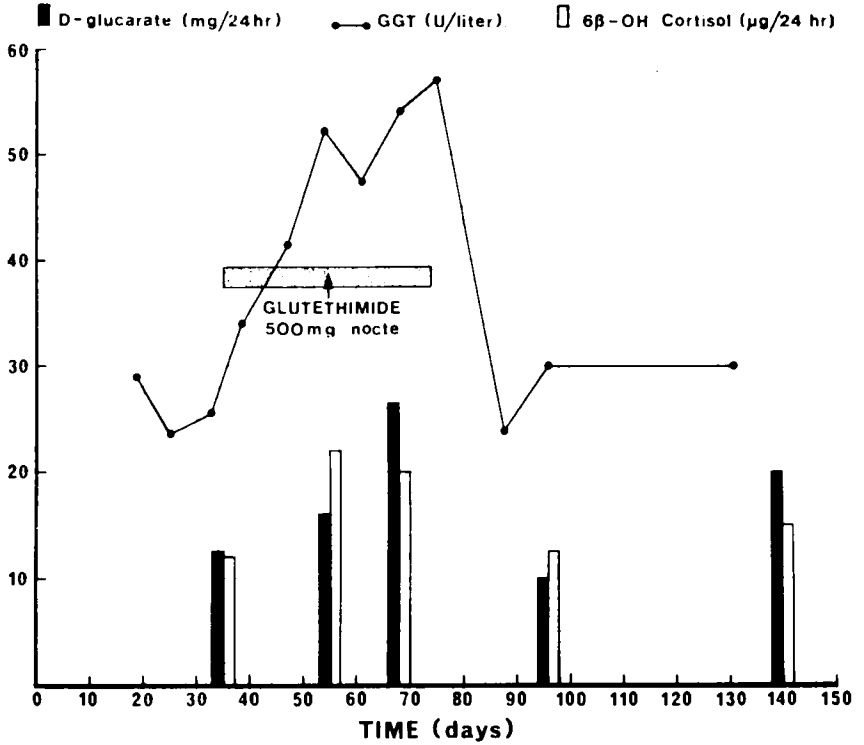


*Figure 1.4. Response of serum γ-glutamyl transpeptidase (GGT) and other parameters of hepatic microsomal enzyme induction (urinary output of D-glucarate and 6β-OH cortisol) to administration of glutethimide in a physically healthy 28-year-old female with anxiety state. (J. V. Martin and G. Silverman, unpublished data)*

one month and reaches a peak 7–11 days after onset of symptoms, the test was proposed as a valuable diagnostic index in detecting cases presenting in the later stages of the disease [432, 486, 487]. On the basis of clinical, experimental and histochemical studies, it has been suggested that, since human myocardium is low in GGT activity, the enzyme may originate from the lysosomes of invading leucocytes or from the capillary endothelium of regenerating blood vessels, both of which histologic features are prominent in necrotic areas of myocardium during the processes of tissue repair and in line with the time-course of serum GGT activity after infarction [488, 489]. A recent report described raised GGT activities in only 54% of infarct cases followed over a 4-week period, and most of these had evidence of hepatic dysfunction [490]. This seems to be supported by the observation of increased $LD_5$ content of serum in infarct patients with raised GGT activity [491], and the hypothesis that the post-infarct increase in GGT originates from liver as a consequence of anoxic damage appears as plausible as the suggestion that it arises as a consequence of repair processes within the myocardium. If the latter view proved correct, the cardiologist would have a valuable prognostic tool at his disposal, since subjects manifesting high levels ought then to make a more complete recovery. No study correlating GGT levels with survival has yet been published.

The situation is indeed becoming more confused consequent upon reports that the incidence of raised serum GGT activity after infarction is less than that found by previous authors. In one study, the incidence was less than 25% [492]. In another, the incidence was 19% in patients without heart failure and 86% in patients with heart failure, when hepatic congestion was presumed to be responsible for the raised GGT [493].

*Specificity*

Apart from those conditions already described, serum GGT elevations occur during rejection of kidney transplants [494] and in certain neurologic diseases [495]. Raised levels are not encountered in diseases of muscle [496], but 3-fold elevations have been reported in 65% of cases suffering from angina and increases up to 6-fold in some diabetics, especially those with vascular complications [471, 478]. GGT is a more powerful tool in excluding desease than in confirming disease, although it can provide the answer to some clearly defined problems so long as its total lack of specificity is recognised. There are few laboratories in the United Kingdom where the test is unavailable, and yet a mere 5 years ago

it was almost unknown. The proper place of this determination is likely to be as part of a profile, especially in conjunction with discriminatory mathematical techniques. The simplified ratio procedures so far tested do not seem to be effective [460, 466].

## GGT ISOENZYMES

Many approaches to the problem of identifying GGT isoenzymes have been used. Initial investigations were carried out by paper electrophoresis, followed by elution of the enzyme from segments of the cut paper [497, 498]. The authors concluded that the absence of organ-specific isoenzymes rendered the procedure unsuitable for clinical diagnosis. Utilising agar gel electrophoresis followed by slicing of the gel into segments and elution of the enzyme, Szewczuk found only a single broad zone of GGT activity in human serum and tissues which migrated between albumin and the $\beta$-globulins; on treatment with neuraminidase, however, the entire enzymatically active zone moved towards the cathode, indicating the probability that GGT is a sialoprotein [442]. Subsequent workers obtained up to 3 bands on agar gels [499]. One band was said to increase only in malignant lesions of the pancreas or biliary tree whether or not jaundice was present, and another in most cases of non-malignant disease. Starch gel electrophoresis and Sephadex G-200 filtration experiments demonstrated up to 7 fractions of differing mobility due, in the main, to differences in the size or weight of the enzyme molecules which might in turn have arisen as a consequence of molecular aggregation or association of the enzyme with various serum protein fractions [500, 501]. Specific distribution patterns were described for complete obstructive jaundice and cirrhosis, but their incidence was not given.

Agarose gel was used as the electrophoretic medium and the results of separation compared with Sephadex G-200 filtration [502]. The former procedure gave two fractions in normal and pathological sera, two fractions in normal urine, and 4 in urine from patients with nephrotic syndrome; the latter procedure yielded 3 fractions in a pathological serum. The authors concluded that GGT heterogeneity could be ascribed to different associations of the enzyme–protein molecules. They subsequently described a more sensitive fluorimetric visualisation procedure [503] but did not report on its clinical utility.

The elevated GGT activity subsequent to myocardial infarction has been characterised [489]. Within the first week, a distinct zone of activity between fast $\alpha_2$-globulin and the $\beta$-globulins appeared, to be followed in

the second and third weeks by a second zone migrating with the β-lipoproteins. Two recent studies employed acrylamide gel [504] and cellulose acetate [505] and revealed up to 7 zones of activity in normal and pathological sera. The former authors concluded that the procedure had no tangible diagnostic value; the latter authors were more optimistic and in particular noted a zone of activity in the $\alpha_2-\beta$ region which was most pronounced in patients with obstructive or drug-induced jaundice. There does not as yet appear to be any solid basis for the routine characterisation of GGT isoenzymes, nor any firm conclusions concerning the factors responsible for their heterogeneity.

## α-AMYLASE (EC 3.2.1.1.; Amylase; α-1,4-glucan-4-glucanohydrolase)

This enzyme has been of interest to clinicians and laboratory workers for almost half a century. The most functionally significant α-amylase is that of the exocrine pancreas. This is secreted into the duodenum where it participates in the hydrolysis of macromolecular carbohydrate foodstuffs such as starch and glycogen. A related enzyme is secreted by the salivary glands, but although it initiates the digestion of carbohydrates, its action is terminated on entry of the food into the stomach where contact is made with HCl and pepsin. These two digestive forms of α-amylase have similar amino acid composition and peptide maps, and each is a single peptide chain, with pancreatic amylase having a molecular weight of 54000 compared with molecular weights of 62000 and 56000 for the A and B forms of salivary amylase respectively [506].

Other α-amylases are found in the small intestine, kidney, testes and fallopian tubes [507–509]. It has frequently been stated that liver is another source of α-amylase. While this is undoubtedly true of the rat [510, 511], the evidence of several investigators indicates that this is not so in the human and that the traces of amylase found in human liver may be due to pooling of blood within that organ [173, 512–514].

### METHODOLOGY

The action of α-amylase upon carbohydrate macromolecules is restricted to hydrolysis of α-1,4-glycosidic bonds. The residual complex therefore preserves its α-1,6-glycosidic links intact and the other products are essentially maltose units. Until recently the available methods for measuring α-amylase activity utilised determination of the rate of appearance of reducing sugar (saccharogenic) or the rate of disappearance of

starch (amyloclastic) as monitored by decolorisation of the blue starch–iodine complex [515]. Improved saccharogenic methods have been presented [516, 517], automated versions of the amyloclastic technique [518], and of the saccharogenic technique [519, 520] have been published.

The most striking methodological development in the past few years has been the synthesis of amylase substrates in which a dye is covalently linked to an insoluble or easily precipitable carbohydrate polymer. These include starch coupled with Remazol Brilliant Blue [521–523]; amylose linked to Cibachron Blue [524–526]; a cross-linked starch derivative coloured by a dye marker and given the commercial trade-name 'Phadebas' [527–529]; amylopectin dyed with Reactone Red 2B [530–534]; and amylopectin bound to Procion Brilliant Red M-2BS [535]. In all the above methods, hydrolysis of the substrate leads to the liberation of water-soluble fragments containing the dye. It is then a simple method to remove the undigested polymer by precipitation or centrifugation and to measure the absorbance of the supernate. Conditions are selected which permit a relationship (linear, curvilinear, or log–linear) between absorbance and enzyme activity such that the latter can easily be derived from the former. These dyed substrates may be incorporated into agar to provide a medium suitable for semi-quantitative amylase assay based on measurement of the area of decolorisation produced by dilutions of the enzyme-containing solution [536, 537], but the practical advantages of these procedures are slight in view of the prolonged incubation times that are necessary and the difficulties in calibration and standardisation. An automated method using the amylose–Remazol Brilliant Blue conjugate has been published [538].

A different approach utilises nephelometry to measure the light-scattering property of a starch substrate solution [539, 540]. This is reduced as hydrolysis of the starch proceeds, and the rate of reduction can be continuously monitored to provide a sensitive kinetic assay of amylase activity. Fluorescence measurements have also been exploited in amylase assays. Linkage of isatoic anhydride to a starch derivative provides a fluorochromatic substrate which has been used in a sensitive automated technique [541]. Use of radioactive $^{14}$C-starch as substrate has allowed development of a very sensitive assay procedure [542].

## CLINICAL SIGNIFICANCE OF SERUM AMYLASE

Historically, the principal application of amylase determinations has been in the diagnosis of acute pancreatitis [543]. This may exist in oedematous

or haemorrhagic forms; the latter carries a more serious prognosis and is associated with more pronounced rises in amylase activity than the former. Regrettably, certain conditions which require to be distinguished from acute pancreatitis are also associated with raised amylase activity, notably perforated peptic ulcer, intestinal obstruction, cholecystitis and ruptured ectopic pregnancy. Administration of analgesics such as morphine may be associated with transient pronounced amylase elevation ascribed to spasm of the sphincter of Oddi [544]. Mumps parotitis or orchitis have also been reported to generate raised amylase activities although confusion with acute pancreatitis is unlikely.

The elevation of serum amylase in acute pancreatitis occurs promptly after the onset of symptoms, reaches a peak within 24 hours, and in uncomplicated cases returns to normal by the fourth day. The rapidity of these changes is such that a diagnostically significant elevation may be missed. In a proportion of cases, no elevation whatsoever is detected. Although no level is absolutely diagnostic of acute pancreatitis, it is generally held that the probability of the diseases increases with the magnitude of the serum amylase elevation [545]. This view has, however, been challenged, and evidence has been put forward suggesting that the degree of hyperamylasemia is inversely related to the likelihood of acute pancreatitis [546]. A 30% incidence of hyperamylasaemia has been reported to follow operations on the biliary tree [547], but a more recent study has assessed the incidence as only 6%, and even then, the hyperamylasaemia was only rarely accompanied by clinical manifestations of pancreatitis [548]. Macroamylasaemia (vide infra) is a rare cause of post-operative elevation of the serum amylase [549]. Two unusual cases have been described in whom hyperamylasaemia was due to ectopic enzyme production by lung cancer tissue [550].

One other condition associated with hyperamylasaemia which has attracted recent attention is diabetic ketoacidosis [551, 552]. Study of an unselected consecutive series of acute admissions revealed that serum amylase was elevated within the initial 24 hours in 60% of the cases; indeed, an elevation exceeding 5-fold the upper normal limit was encountered in 17% of the cases [553]. There was no association between hyperamylasaemia and clinical or laboratory features suggestive of acute pancreatitis, but there appeared to be a relationship with the extent of the hyperglycaemia. This observation would accord with an earlier report defining a relationship between the serum amylase and serum osmolality in diabetic coma [554]. It has been suggested that the amylase in such cases is released from hepatic microsomes consequent upon activation of

lysosomal hydrolases [555], but this hypothesis seems untenable in the light of clinical and experimental studies establishing a negative gradient for amylase between liver and plasma, and normal values for plasma amylase in subjects with acute liver necrosis [173]. Indeed, serum amylase activity is reduced in most patients with liver disease [556], and the immediate effect of hepatectomy in the dog is a rise in the serum amylase, which later returns to normal [557]. In the rat, however, electrophoretic studies strongly suggest that the serum amylase originates from the liver [558]. It has been proposed, as an alternative explanation, that amylase is released from the pancreas as a consequence of dissociation of pancreatic zymogen granules and altered cell membrane permeability arising from the severe metabolic disturbance.

If the specificity of serum amylase elevations for acute pancreatitis is in doubt, there is a fair measure of agreement that in the presence of acute pancreatitis, prolonged and persistent elevations of serum amylase herald the onset of complications such as pseudocyst formation [559, 560].

Another role for serum amylase assay concerns the response of the pancreas to stimulation by the entero-hormones secretin and pancreozymin [561]. Normal individuals show little change. When the pancreatic duct is occluded by stone or by tumour with the bulk of the organ uninvolved by the disease process, a sharp increase in serum amylase may be detected. As the acinar tissue is destroyed, be it by malignancy or consequent upon chronic pancreatitis, the response becomes progressively smaller, and when sufficient of the organ has been destroyed as to render the patient diabetic, the response is negligible. These findings have been confirmed by various authors [562–564], but when stimulation of the pancreas is by means of a test meal, the serum amylase response does not allow adequate discrimination between subjects with pancreatic disease and controls [565].

## URINE AMYLASE

The renal clearance of amylase is directly proportional to the creatinine clearance and is about 3% of the glomerular filtration rate in man [566]. In patients with normal renal function, urine amylase excretion is therefore proportional to the serum amylase activity. Urine amylase will therefore be increased whenever the serum amylase is raised. The role of urinary amylase determinations is not clearly established, partly because there is no unanimity as to whether measurement should be as a concentration, as an output, or as an excretory rate. Gambill and Mason [567] advocated the

one-hour excretion rate of urinary amylase as the most accurate diagnostic index of acute pancreatitis (*Figure 1.5*), and they also found it to be a valuable aid in the detection of pancreatic carcinoma [568], Support for the former view is provided by the data of Calkins [569], but in a more recent study, the authors found serum lipase more accurate than the one-hour urinary amylase excretion in diagnosing acute pancreatitis [570]. Evidence has been presented that the 24-hour urinary amylase excretion is directly related to the functional state of the pancreas, being reduced when the gland is chronically diseased [571].

Various measurements of urinary amylase have been made following pancreatic stimulation and appear to provide a useful index of pancreatic function [572]. The output 2 hours after secretin stimulation correlated well with duodenal bicarbonate concentration, both being reduced in chronic pancreatic disease [573]. However, stimulation must be submaximal to achieve optimal discrimination between subjects with pancreatic disease and normal controls [574].

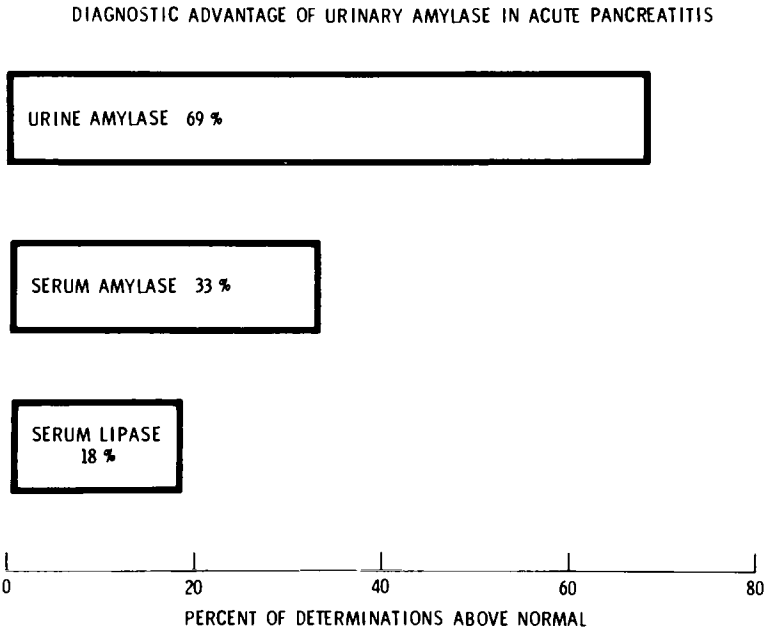


*Figure 1.5. Relative percentage incidence of abnormal values for urine and serum amylase and serum lipase in acute pancreatitis [6]*

## MACROAMYLASAEMIA

In 1967, Berk, Kizu, Wilding and Searcy described elevation of serum amylase activity due to a circulating high molecular weight protein [575]. The disorder was found in alcoholics or subjects with abdominal pain, but these features prompted the investigation in the first place; there is no evidence that specific clinical features and the macroamylasaemia are necessarily related, and measurement of serum lipase and direct tests of pancreatic function have consistently yielded normal results in such patients [576]. The clearance of amylase relative to that of creatinine is greatly reduced in subjects with macroamylasaemia in contrast to renal disease, where both clearances are reduced in parallel, and acute pancreatitis where the amylase clearance is greatly increased [577]. Although the condition will usually be suspected in subjects manifesting raised serum amylase and low urinary amylase in the presence of normal renal function, as the large molecule cannot pass through the glomerular membrane, these criteria in themselves are unreliable, since the presence of salivary-type amylase in serum results in a similar pattern [578]. For this reason, reliable screening procedures are of great diagnostic value in recognising this condition. One such test is based upon a small-column gel filtration procedure [579]. Another utilises thin-layer gel filtration [580]. The fact that the activity of macroamylase increases with rising temperature more steeply than that of normal serum amylase provides the rationale for a third test [581].

While the electrophoretic properties of macroamylase studied by paper electrophoresis were not distinguishable from those of normal serum amylase [582], ultracentrifugation showed major differences between normal amylase and macroamylase [583]. Indeed the latter was not a homogeneous protein, but a family of proteins whose sedimentation constants range from 7.75 to greater than 19.5. For this reason, it is probable that the complex arises from binding of a normal amylase component to several macromolecular species present in tissues or plasma. Evidence is forthcoming that immunoglobulin A [584], and glycogen [585, 586] may be responsible. It has also been shown that more salivary-type than pancreatic-type amylase is present in naturally-occurring macroamylase complexes [587]. This is because the agents responsible have greater binding affinity for salivary-type amylase and not because the latter is more readily available in the plasma of affected subjects [588]. The binding agents apparently are capable of distinguishing to some extent between amylase proteins of different species [589].

## ISOAMYLASES

The heterogeneity of amylase isoenzymes in human tissues and fluids has been documented in various publications over the past decade which have been reviewed by Meites and Rogols [590]. The degree of resolution obtained by different authors has been extremely variable, depending upon whether gel filtration, column chromatography, or zone electrophoresis was the technique employed, and, in the latter instance, upon the choice of buffer and support medium. Using electrophoresis in polyacrylamide gels, human salivary and pancreatic amylase could be separated into 5 and 6 different zones respectively with only limited overlapping although occasionally an additional 2 zones could be detected in saliva [591–593]. However, immunochemical investigations demonstrated no difference between human salivary and pancreatic amylases in one study [594] and only minor differences in another study [595].

Several groups have obtained evidence for the occurrence of two zones of amylase activity in human serum corresponding to the mobility of human pancreatic and salivary amylases [596–598]. The pancreatic-like isoenzyme of serum was resolved into two bands [599], and it was subsequently reported that the salivary-like isoenzyme was likewise resolved into two bands [600]. Isolation of pancreatic-like and salivary-like amylases from human serum and urine was also achieved by means of gel filtration and ion-exchange chromatography [512], and clinical studies established the pancreas as the probable source of pancreatic-type amylase in human serum; other human tissues contained amylases with chromatographic properties similar to those of pancreas and salivary glands. However, a detailed analysis of human tissue amylases using agar and polyacrylamide gel electrophoresis showed such overlap between the various tissues that the authors drew no firm conclusions on the origin of human serum amylase [601].

The clinical utility of isoamylase determination has not so far proved impressive. It has been suggested that a relative absence of pancreatic isoenzymes occurs in the serum of patients with cystic fibrosis and reflects decreased pancreatic function in this disease [602]. The most authoritative clinical studies have been conducted by Aw and Hobbs, and have concerned urine rather than serum isoamylases [603, 604]. The isoenzymes defined by these authors are shown in *Figure 1.6.* In 90% of normal subjects, only $P_2$ and $S_1$ are found, and irrespective of the isoamylase pattern, those presumed to be of pancreatic origin predominate. In mumps, however, salivary isoamylases became dominant due to a

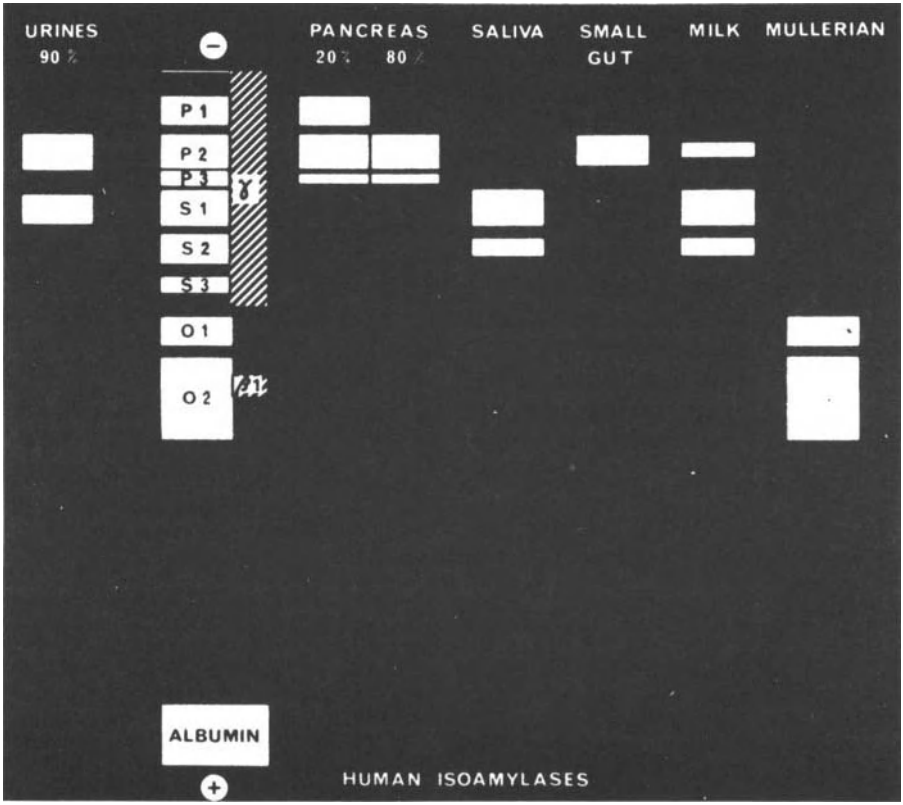


*Figure 1.6. Human isoamylases detectable on agar gel electrophoresis, compared with albumin, β′-globulin and γ-globulin zones in same system [604]*

relative increase, and in pancreatic insufficiency they became dominant because of a relative decrease in the pancreatic isoamylases present in the urine; the authors stressed the utility of this latter observation which they found to hold for 94% of their patients with chronic pancreatitis.

Until recently, virtually all investigators utilised a starch–iodine technique for visualisation of the isoamylases after electrophoretic separation. This is very prone to interference by the plasma proteins which interact with iodine and simulate amylase activity [605]. For this reason, the chromogenic substrates described earlier are finding increasing application in the identification of isoamylases, notably the 'Phadebas' derivative [606, 607], and Remazol Brilliant Blue starch [608, 609]. By comparing the ratios of the activities obtained using soluble starch and an insoluble dyed

starch as substrates, it is possible to distinguish amylase of pancreatic and salivary origin since the latter yields a much higher ratio [610].

## LIPASE (EC 3.1.1.3; glycerol ester hydrolase)

This enzyme hydrolyses triglycerides. Pancreatic lipase is of great clinical interest and its mechanism of action has been extensively studied [611]. Hydrolysis commences rapidly at the outer ester linkage. The resulting diglyceride is susceptible to further hydrolysis at the remaining external linkage, leaving a monoglyceride esterified on the $\beta$-carbon. This is resistant to lipase, but such monoglycerides may undergo isomerization with the fatty acid being transferred to one of the two external hydroxyl groups; in this event, hydrolysis to free glycerol can then take place. The products are therefore mainly monoglycerides and free fatty acids, together with some diglycerides and a little glycerol. A number of features distinguish this enzyme. It acts primarily at the interface of an aqueous triglyceride emulsion. A protein cofactor, called colipase, is required for full activity. Bile salts activate the enzyme in the presence of colipase and inhibit the enzyme in its absence [612, 613]. Little is known about the presence of true lipase in human tissues other than the pancreas, and the search for such knowledge would have to contend with the reality that most such tissues contain a variety of non-specific esterases capable of mimicking certain actions of lipase.

### TECHNIQUES OF ASSAY

In the historical assay of Cherry and Crandall [614], oleic acid released from triolein is titrated with alkali, but a 24-hour incubation is needed for serum. A standard method has been proposed, based upon the above chemistry, but using an automatic pH-stat titrator to add the alkali and generate the progress curve of the reaction over a measuring period of 3–8 minutes [615]. The supply, draining and rinsing of the pH-stat cell can be automated by sequential flow, but the system operates at a rate of only 6 estimations per hour [616].

A number of methods have been based upon extraction of the free fatty acids which may then be measured as copper soaps [617, 618] or by direct reaction with diethyl dithiocarbamate [619]. The extracted fatty acids may also be measured by a sequential flow analysis system in which they are made to change phenol red to a yellow colour [620]. The problem with these methods is to obtain and maintain the substrate free of fatty

acids. α-Naphthyl palmitate has been introduced as a substrate, since pancreatic lipase, but not other esterases, can hydrolyse the compound to release α-naphthol which can then be coupled with Fast Violet B to yield an easily measured diazo compound [621].

Fluorometric methods have also been presented. A manual assay is based upon use of the dilauric ester of fluoresceine; hydrolysis by lipase liberates free fluoresceine [622]. A similar compound, monodecanoyl fluoresceine, has been used as substrate in an automated method [623]. A recent innovation has been the development of methods which monitor the decrease of turbidity of a stable triolein emulsion consequent upon hydrolysis of the lipid micelles [624, 625]. These seem to be an improvement upon an earlier procedure utilising a similar principle [626].

## CLINICAL APPLICATIONS

Opinions concerning the value of serum lipase estimations in acute pancreatitis have until recently been prejudiced by the necessity for a prolonged incubation period or the tediousness of many of the available techniques. Worse still, many reports were based upon fallacious methods using non-specific substrates readily hydrolysed by esterases other than lipase. Gambill and Mason [567] detected elevated serum lipase in only 18% of cases of acute pancreatitis, as shown in *Figure 1.5,* but Tietz, Borden and Stepleton [627] had earlier shown a greater increase in serum lipase than in serum amylase among 35 patients with established pancreatitis. The diagnostic superiority of lipase over amylase in acute pancreatitis seems to be increasingly established [570, 628–630], especially as regards the early stages of the disease. For example, Waller and Ralston [570] obtained an incidence of 89% raised lipase and only 32% raised amylase in their cases. The improved specificity of lipase for pancreatic disease has been noted [631], and it seems a safe recommendation that wherever possible, both amylase and lipase activity of serum should be measured in acute abdominal emergencies suggestive of acute pancreatitis. It cannot be emphasised too strongly that methods for lipase based upon aqueous substrates may lead to clinically erroneous conclusions [630].

Despite early reports that serum lipase may be raised [632] or reduced [633] in pancreatic carcinoma, the changes do not seem sufficiently consistent to warrant diagnostic use [634]. Increased serum lipase has been reported to follow severe bone fractures, highest values being found when fat embolism occurs [635, 636].

Urinary lipase excretion has been measured by some investigators. It

does not seem to be of value in acute pancreatitis [571], but a number of reports claim that values are low in patients with chronic pancreatitis or pancreatic carcinoma and fail to increase after secretin administration [637–639].

## LACTATE DEHYDROGENASE (EC 1.1.1.27.; LDH; L-Lactate: NAD oxidoreductase)

This zinc-containing enzyme is widely distributed throughout all body tissues where it is present predominantly in the cytosol. By regulating the interconversion of lactate and pyruvate, it exercises a control function over the balance between respiration and glycolysis [640]. Consistent with this key regulatory role is the fact that the enzyme consists of tetramers derived from 2 polypeptide chains which show marked differences in substrate affinity and kinetic behaviour. Recognition of the molecular heterogeneity of LDH gave birth to the concept of isoenzymes which was subsequently found to have wide application to many other enzyme systems. Initial studies with total LDH activity reported its usefulness in the diagnosis of myocardial infarction, hepatic disease and malignant disease, but a growing awareness of the non-specificity of abnormal total LDH levels has prompted a reaction in favour of LDH isoenzyme determinations. Even so, analysis of LDH isoenzymes, apart from its inconvenience and the compromises involved in most of the techniques, does not seem capable of solving many clinical problems which are not amenable to more simple procedures.

### TECHNIQUES FOR TOTAL LDH ASSAY

#### *Spectrophotometric methods*

The enzyme catalyses the following reversible reaction:

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{lactate} + \text{NAD}^+$$

The reaction can be followed in either direction by monitoring the change in absorbance at 340 nm due to alteration in the redox state of the pyridine nucleotide. The equilibrium strongly favours lactate formation, and this principle was used in the first clinical assay [641]. The alternative approach, starting with lactate, has been advocated although the reaction rate is much lower [642, 643]. An advantage of the latter lies in the fact

that it is generally more satisfactory to measure an increase than a decrease in absorbance. A serious difficulty of the method starting with pyruvate is that the $K_M$ of heart muscle LDH for this substrate is low, and the enzyme is highly susceptible to substrate inhibition; the $K_M$ for skeletal muscle and liver LDH is much higher and substrate inhibition requires excessive amounts of pyruvate [644–646]. For this reason, assays based upon pyruvate as the initiating substrate have to strike a delicate balance between utilising a concentration optimal for heart and suboptimal for liver and muscle LDH, or more nearly optimal for the latter two with consequent inhibition of heart LDH [647]. Increase in temperature of the reaction also changes substrate affinity of LDH, and this interdependence is more critical than for any other clinically-important enzyme [648–650]. Gay, McComb and Bowers carried out a detailed study of the enzyme reaction and the conditions generating optimal activity in each direction; they concluded that, using these conditions, it mattered little from an analytical or clinical viewpoint whether lactate or pyruvate was the chosen substrate [651]. Other authors, who performed a clinical evaluation of 4 different methods, were not in agreement, and claimed that the 'pyruvate-to-lactate' reaction was clinically and methodologically less desirable [652].

Nevertheless, the 'pyruvate-to-lacate' reaction remains, with all its imperfections, the most popular spectrophotometric LDH assay. An instrument interface system linked to a computer [653] and a rapid monitoring system to simplify its determination [654] have been described; it has also been adapted for use with a commercial reaction rate analyser [655] and with the centrifugal fast analyser, where a non-linear regression equation apparently yields more accurate results than the conventional linear regression approach [656]. A less sophisticated procedure was advocated by Fasce and Rej [657] who measured the reduction of fluorescence, rather than absorbance, of NADH during LDH action and used a sequential flow split-stream technique to provide 3 estimates at timed intervals. Martinek [658], while retaining absorbance measurement, used a batch technique in preference to continuous monitoring, stopping the enzyme reaction with a borate buffer. In an attempt to accelerate the 'lactate-to-pyruvate' reaction and extend its range of linearity, semicarbazide has been advocated as a trapping agent [659].

*Colorimetric methods*

The earliest methods of this type used 2,4-dinitro-phenylhydrazine to couple with pyruvate, the disappearance of which was measured in one

method [660], whereas its appearance from lactate was measured in another [661]. It is now known that NADH reacts with 2,4-dinitrophenylhydrazine to produce a compound absorbing at the same wavelength as the pyruvate complex [662]. An automated colorimetric assay is based upon reduction of ferric to ferrous ions by NADH generated from lactate [663]; the ferrous ions are complexed with 2,2-bipyridyl to yield a coloured derivative according to a reaction first described by Whitaker [664].

The use of tetrazolium salts as electron acceptors for the estimation of LDH when lactate is the substrate has become well established as a staining procedure for LDH isoenzymes; diaphorase or phenazine methosulphate facilitate the electron transfer process, and the colourless tetrazolium is reduced to an insoluble formazan dye. Methods are available to stabilise the dye as a colloid, and the colour, which can be measured photometrically, is proportional to enzyme activity [665, 666]. The commonest tetrazolium for this particular application is INT (2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride), and the method lends itself to automation [667]. A recent development has been the introduction of kinetic tests based upon the formation of this stabilized formazan [668, 669]. The methods yield problems with blank reactions, but the molar extinction coefficient of the formazan is three-fold that of NADH, and the electron transfer step pulls the reaction in the direction of NADH formation.

## LDH ISOENZYMES

### *Chemical nature*

Each isoenzyme of LDH comprises a tetramer. The subunits consist of two polypeptide chains – the H chain characteristic of heart muscle LDH, and the M chain typical of hepatic and skeletal muscle LDH. Each polypeptide is the product of a single gene locus, and polymorphism may occur at one or other locus to yield up to 15 distinct bands on electrophoresis [670, 671]. Normally, however, up to 5 may be recognised in tissues and serum. They are numbered from $LD_1$ to $LD_5$ in order of their rate of migration towards the anode. $LD_1$ has the composition $H_4$ and $LD_5$ the composition $M_4$. The intermediate isoenzymes have the following composition: $LD_2$, $H_3M$; $LD_3$, $H_2M_2$; $LD_4$, $HM_3$. Freezing and thawing a mixture of equal parts of the pure $LD_1$ and $LD_5$ isoenzymes yields a mixture of all 5 consistent with random combination of the subunits [672], as shown in *Figure 1.7.* There is evidence, however, that in vivo

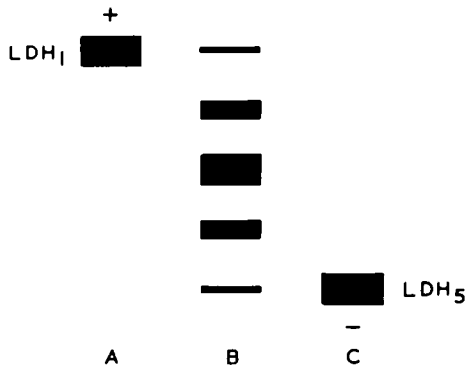


*Figure 1.7. Effect of freezing and thawing a mixture of pure lactate dehydrogenase isoenzymes demonstrated by starch gel electrophoresis [671]. (A): Pure $LD_1$. (C): Pure $LD_5$. (B): Mixture of A and C after freezing and thawing*

combination is much more complex and is subject to genetic regulation [673, 674]. Mention must be made of a sixth isoenzyme, $LD_x$, which has been identified in the testes and sperm of human subjects [675, 676] and of other species [677]. Experimental studies have shown that a specific antibody to $LD_x$ reduces fertility in the mouse [678].

A number of abnormal LDH isoenzyme bands have been detected in human serum and have been shown to be due to factors other than genetic polymorphism. One report has documented the presence of a macromolecular LDH complex in serum of a patient with leukaemia [679]; interaction between LDH and immunoglobulin G has also been described [680]; and several papers describe complexing of LDH isoenzymes with immunoglobulin A [681–685]. Other aberrations reported include a duplet form of $LD_1$ in a haemophilic patient given a large injection of cryoglobulin precipitate [686]; this persisted only for a few days. A family has been described with one member homozygous and a number of members heterozygous for a deficiency of the H polypeptide of LDH [687]. An additional band, named by the authors LDH-T, appeared between $LD_4$ and $LD_5$ after acrylamide gel electrophoresis in a high proportion of patients with jaundice due to severe liver disease, most of whom succumbed within a few weeks of the band being identified [688]; unfortunately, the nature of the abnormality was not ascertained, and an incidence of that magnitude has not been reported for any similar LDH isoenzyme variant in liver disease.

*Techniques of separation*

Of the methods which have stood the test of time, those using cellulose acetate as separation medium remain popular [689–692]. Agar gels [693–697] and agarose films [698] have their advocates. Starch gels were used in many of the pioneer studies on LDH isoenzymes [699–702]; this still remains a powerful technique for definitive and reference analyses, although the difficulties in quantitation it presents detract from its popularity in routine laboratories. Polyacrylamide gel electrophoresis of LDH is now making a considerable impact [703, 704], and isoelectric focusing in acrylamide has also been reported [705].

Once separated, the isoenzymes may be assayed following elution, or they may be assayed in situ using a substrate-impregnated agar overlay and monitoring the absorbance at 340 nm [706]. The commonest method, however, involves use of tetrazolium salts to act as electron acceptors with generation of insoluble formazan dyes, as described previously. Hydrazine or cyanide can be used to trap the pyruvate formed from lactate and thus accelerate the reaction in the desired direction. The resulting bands may be scanned and integrated [695, 707, 708]. More sophisticated techniques for quantitating individual isoenzyme activity have also been presented [709, 710]. Elution of the formazan dye from the gel and its quantitation by colorimetry can also provide a measure of isoenzyme distribution [711].

For all their elegance and the ingenuity which has gone into their development, electrophoretic techniques have several disadvantages. A survey of data obtained during studies of human tissue and serum LDH isoenzymes using various techniques is assembled in *Table 1.5* which is drawn from the review by Roman [712]. Different distributions are given by different electrophoretic media in the same tissue, and studies on the same tissue by different workers using the same medium often reveal disagreement. Some of these problems can be attributed to artefactual bands on the gel appearing in the absence of substrate. These have been attributed to alcohol dehydrogenase acting upon traces of ethanol present in the reagents [718, 719]. It has also been suggested that this activity arises from the action of LDH on traces of hydroxy-acids to be found in gel media [720]. Other sources of concern include the wide range of reaction conditions used by various authors for carrying out the visualisation procedure and which in many instances are far from optimal [721]. The lack of quality-control procedures has until recently been another drawback, although a useful system has now been proposed [722].

Table 1.5. DISTRIBUTION OF LDH ISOENZYMES IN HUMAN TISSUES AND FLUIDS [712]

| *Tissue* | *LDH Isoenzymes as percentage of total* | | | | | *Method of separation* | *Reference* |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | | |
| Heart | 49 | 48 | 3 | 0 | 0 | Starch gel | 701 |
| | 35 | 26 | 12 | 16 | 11 | Agar gel | 713 |
| | 40 | 32 | 26 | 0 | 0 | Agar gel | 714 |
| | 57 | 32 | 7 | 2 | 2 | Cellulose acetate | 715 |
| Liver | 2 | 5 | 12 | 0 | 81 | Starch gel | 701 |
| | 2 | 4 | 11 | 27 | 56 | Agar gel | 713 |
| | 6 | 8 | 17 | 15 | 54 | Paper | 716 |
| Kidney | 30 | 50 | 15 | 5 | 0 | Starch gel | 701 |
| | 12 | 14 | 24 | 25 | 23 | Agar gel | 713 |
| | 29 | 28 | 30 | 12 | 1 | Paper | 716 |
| Muscle | 16 | 18 | 18 | 12 | 36 | Starch gel | 701 |
| | 4 | 7 | 21 | 27 | 41 | Agar gel | 713 |
| | 5 | 7 | 12 | 19 | 58 | Agar gel | 717 |
| | 1 | 2 | 34 | 30 | 33 | Paper | 716 |
| Serum | 39 | 46 | 11 | 2 | 1 | Agar gel | 717 |
| | 39 | 37 | 17 | 4 | 4 | Agar gel | 694 |
| | 30 | 40 | 20 | 6 | 4 | Agar gel | 695 |
| | 33 | 45 | 19 | 3 | 1 | Polyacryl-amide | 703 |
| | 31 | 39 | 19 | 6 | 5 | Cellulose acetate | 690 |

### *Chemical identification of isoenzymes*

Because of the above difficulties in quantitating LDH isoenzymes and the need for a large throughput in routine laboratories which electrophoretic techniques are not intrinsically suited to meet, much attention has been devoted to exploiting chemical and physical differences between the pure tetrameric forms, $LD_1$ and $LD_5$. Use of chloroform [723] and acetone [724] to inactivate all fractions except $LD_1$ were of interest but necessitated centrifugation to remove denatured protein prior to carrying out the assay.

Oxalate inhibits $LD_1$ and has much less effect on $LD_5$ [725, 726]. Oxamate likewise inhibits human heart extract by 50% and human liver extract by only 20% [727]. Neither inhibitor is sufficiently specific to

provide clear-cut diagnostic discrimination. The most useful agent in this regard is urea, which inhibits $LD_5$ more powerfully and in lower concentration than $LD_1$ [726, 728–730]. In fact, under certain circumstances, it can be shown to activate $LD_1$ [731]. Using lactate as substrate, Babson [732] developed a diagnostic test for increased $LD_1$ activity, based on a colorimetric LDH assay. This was shown to correlate reasonably well with electrophoretic analysis [733–735], and to provide clear-cut distinction between subjects in whom LDH of myocardial or of hepatic origin predominated [736]. An alternative method utilising pyruvate has been presented [737], and a detailed study of the interrelationship of urea and pyruvate concentrations has been conducted at 25°C [738] and at 37°C [739]; the latter author, in particular, claimed to obtain complete separation of patients with myocardial infarction and those with various liver diseases by his recommended procedure.

*Heat stability*

The H polypeptide of LDH is much more stable on heating than is the M polypeptide. Consequently, various attempts have been made to exploit this difference to determine the predominant isoenzyme component in clinical samples. The 'heat-stable fraction' has been determined after heating at 65°C [740, 741], at 60°C [742], and at 58°C [743]. An ambitious scheme for separating $LD_{1+2}$, $LD_3$, and $LD_{4+5}$ based on heat inactivation at 3 different temperatures has been proposed [744], and an automated assay has been presented [745]. The test seems to be widely accepted as an improvement upon assay of total LDH activity in the diagnosis of myocardial infarction [746, 747]. Sinha and Trew recommended it as a screening test rather than as a definitive procedure [748]. Auvinen and Konttinen, on the other hand, carried out a detailed study, comparing the clinical utility of heat-stable LDH, urea-stable LDH, and analysis of LDH isoenzymes using agar gel electrophoresis [749]. They concluded that the former two procedures were more reliable and accurate in confirming myocardial damage than electrophoresis, with heat-stable LDH having a slight margin of superiority. This opinion is shared by more recent authors [750]. Technically, however, the urea-stable LDH is easier to estimate because urea concentrations can be maintained more reproducibly than can temperature; a waiting period is not necessary; and turbidity does not develop as occasionally happens after heating due to protein denaturation.

*Differential substrates*

In addition to pyruvate, a number of other oxo-acids can act as substrates for LDH and undergo reduction in the presence of NADH. The first to be introduced to clinical use was 2-oxobutyrate which is reduced by LDH to 2-hydroxybutyrate. This activity was given the nomenclature '$\alpha$-hydroxybutyrate dehydrogenase' by the authors who first reported its significance [751]. The early literature documented the value of this enzyme in diagnosing myocardial infarction but also recorded raised activities in a proportion of patients with liver disease; for this reason, the ratio of LDH to HBD activity was proposed as a means of improving discrimination between these disease states, higher values being found in patients with liver disease and lower values in patients with myocardial infarction [752, 753].

Although Rosalki and Wilkinson [751] selected a concentration of 3.3 mmol/litre 2-oxobutyrate for their assay at 25°C because they considered it to be optimal for human serum, Rosalki later admitted that this was suboptimal but gave the best possible discrimination between $LD_1$ and $LD_5$ [754]. The use of the LDH/HBD ratio was also based upon the same temperature and substrate concentration [753]. With the advent of automatic reaction rate analysers operating at higher temperatures, optimal HBD assays at 37°C were presented [649, 755]. These utilised 15 mmol/litre 2-oxobutyrate. It was claimed by Smith [648] that when optimal methods were employed at 37°C, the diagnostic discrimination afforded by the LDH/HBD ratio was lost. This was subsequently confirmed [650], but optimal 2-oxobutyrate concentration was preferred as the diagnostic value of the HBD assay per se, although not the LDH/HBD ratio, was undiminished utilising these conditions. A comparative evaluation of the original assay at 25°C [751] and the revised assay at 37°C [755] showed that such clinical advantage as existed lay with the revised assay which, additionally, was analytically superior to the original assay [756]. A recent report has indicated that use of an optimal 2-oxobutyrate concentration at 30°C (12 mmol/litre) actually enhances the diagnostic value of the HBD/LDH ratio [757].

More recently, other oxo-acids have been evaluated as substrates for LDH. Hydroxypyruvate can undergo reduction to glycerate in the presence of NADH and LDH [758]. The activity, which is referred to as 'glycerate dehydrogenase', can be readily monitored by reaction rate methods, and the substrate, like 2-oxobutyrate, has a higher affinity for the H than for the M polypeptide [759]. The normal range for this activity

is higher than when pyruvate or oxobutyrate is the substrate [760], and it has been shown to be at least as useful as HBD activity in the diagnosis of myocardial infarction [761], although the authors did not examine the incidence of abnormal values in other conditions where false-positive values might have been expected.

The reduction of glyoxylate by human tissues and serum has also been described, the product being glycollate, and the activity described as 'glycollate dehydrogenase' [762, 763]. Although an enzyme glyoxylate reductase (glycollate:NAD oxidoreductase, EC 1.1.1.26) is found in plants, it has not been characterised from mammalian sources, and forceful evidence was presented to implicate LDH as the enzyme responsible for this activity in human serum. In fact, King [764] has shown that the highest rate of activity for LDH occurs when glyoxylate is the substrate used in the assay. This feature is demonstrated in *Figure 1.8*,

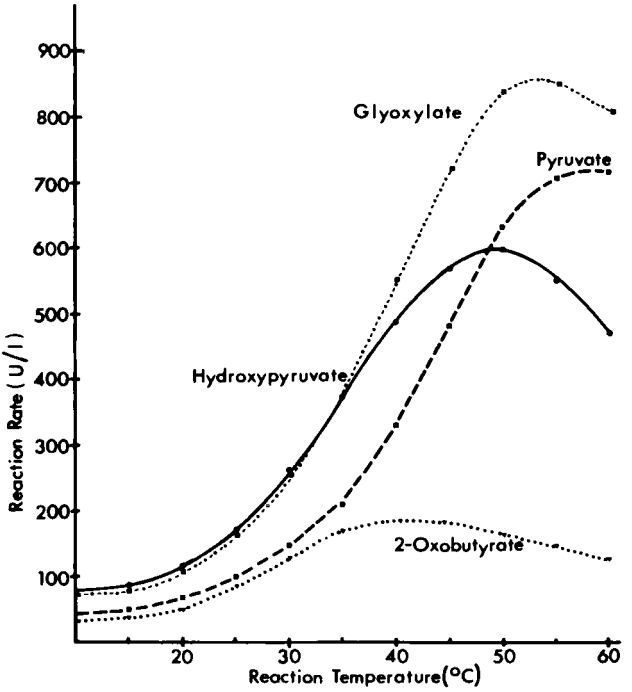


*Figure 1.8. Relative activities of normal human serum lactate dehydrogenase with different substrates at varying temperatures [764]*

which also displays the remarkable sensitivity of LDH to temperature as well as to substrate. A clinical evaluation of 'glycollate dehydrogenase' has been conducted, and the authors have established that the assay has maximum utility when combined as an activity ratio with one of the other oxy-acids [765].

## CLINICAL IMPORTANCE OF TOTAL LDH AND LDH ISOENZYMES

Although total LDH activity of serum was among the first tests to achieve diagnostic significance when the modern era of clinical enzymology was ushered in during the 1950s, the estimation has suffered a decline in popularity due to its lack of specificity. This has only been partially offset by interest in LDH isoenzyme analysis as simpler and reproducible methods for carrying out the latter procedure became available. This section attempts to summarise the more important observations in this field, and further mention of LDH and its isoenzymes are made in the section dealing with myocardial infarction (page 81).

### *Pulmonary disease*

It was reported that raised LDH and normal aspartate aminotransferase activities were consistently found in the serum of patients after pulmonary infarction [766]. Amador and Potchen found LDH to be raised in 40 out of 41 patients in whom the diagnosis of pulmonary embolus was confirmed by lung scanning [767]. Lower incidences have been reported by other authors, ranging from 30–80% [768–770], and it is doubtful if nowadays much reliance is placed upon the test.

Just as the status of total LDH in pulmonary embolism and infarction is controversial, so too is the position concerning LDH isoenzymes. Some workers have described an increase in $LD_5$ activity as the predominant abnormality [769, 771]. Others have found $LD_3$ [695] elevation to be the principal feature. It now seems clear that in clinical subjects the $LD_5$ elevation is primarily due to hepatic necrosis secondary to venous congestion and is often seen when pulmonary embolus is not accompanied by clear evidence of pulmonary infarction, whereas infarction without venous congestion results in raised activities of $LD_1$ and $LD_2$, as well as of $LD_3$ [714, 772]. On the other hand, an experimental study in dogs revealed an identical isoenzyme pattern in animals with pulmonary embolus resulting in infarction and those with pure hepatic congestion [773].

Total serum LDH activity is increased in patients with primary or metastatic lung cancer, the incidence being variously estimated between 25% and 80% [774–776]. An analysis of the isoenzyme distribution in the tumour tissue and in the serum revealed 3 patterns: (a) isolated increases in $LD_3$ which regressed after therapy, (b) enrichment of plasma with tumour isoenzymes which declined when therapy resulted in tumour regression, (c) generalised elevation of all isoenzymes, carrying a poor prognosis, and usually occurring shortly before the demise of the patient [776].

Wroblewski [777] had commented on the usefulness of simultaneous determination of LDH activity in serum and pleural effusion of subjects with the above condition, believing that the serum activity was always higher in non-malignant diseases whereas the effusion always yielded higher activity in primary or metastatic lung cancer. This claim has not been confirmed by other authors who did, however, find increased activity of $LD_4$ and $LD_5$ in malignant effusions which subsequently fell after therapy [778]. This isoenzyme pattern was not exclusive to malignant effusions and was also associated with certain forms of tuberculosis [779].

Increased LDH activity was observed in the sputum in chronic obstructive lung disease [780]. The predominant isoenzymes were the fast-migrating variety (H-type), and the authors attributed their presence in the sputum to its content of polymorphonuclear leucocytes.

*Renal disease*

LDH activity can be determined in the urine when the latter is dialysed to remove inhibitors of the enzyme. With this technique, many authors have demonstrated considerable elevation of the urinary excretion of LDH in patients with renal carcinoma, the incidence of abnormalities being around 90% and unrelated to pyuria, haematuria, proteinuria, or the presence of metastases [781–787]. A correlation with pyuria and proteinuria has, however, been claimed by other workers [788, 789], and this seems to be supported by claims that the isoenzyme distribution of urinary LDH in renal cancer patients is similar to that shown by the cellular elements of the urinary deposit [790, 791].

Several groups of authors have reported unfailing elevation of urinary LDH excretion in subjects with carcinoma of the bladder [781, 787, 790]. Others have found the test raised in only a proportion of such cases [792, 793] or too non-specific to be of value [789], but these studies can be

criticized because the first group used stored urine and inferior assay techniques, while the second group collected many of their samples shortly after cystoscopy. A later report claimed that the test correlates with the presence of pyuria and haematuria rather than reflecting the presence of a bladder tumour as such [794].

There is nothing specific to cancer about urinary LDH elevation, since raised output is also seen in patients with glomerulonephritis [795–797]. These elevations are encountered in the active stage of acute glomerulonephritis, but values decline when the disease process heals and the estimation is therefore useful in assessing prognosis in this disease. Raised urinary LDH activity is also common in chronic glomerulonephritis. No correlation with histologic type, the degree of uraemia, or the urinary cellular content was demonstrated [796, 798]. The test therefore seems to be useful in detecting glomerulonephritis when evidence of other urinary abnormalities or of nitrogen retention is lacking.

Raised urinary LDH levels were noted in 31 of 36 patients with pyelonephritis by Brenner and Gilbert [799]. Other authors have reported a similar incidence (15 of 18 patients) and observed this abnormality in mildly-affected subjects, including some with normal blood urea and urinary bacterial count [796]; since the test gave normal results in patients with acute cystitis only, it can be used to assess the upward spread of urinary tract infection in such cases.

The behaviour of total serum LDH activity in chronic renal failure is controversial. In one report, an incidence of 77% abnormal results during 486 determinations in 76 patients was found, but an automated colorimetric method prone to chemical and drug interference was used, and no indication was given as to how abnormal the results actually were [800]; it was also stated that a significant further rise took place in 17 patients subjected to multiple haemodialysis totalling 48 individual procedures. Other authors found no significant change in serum or red cell LDH activity in 75 uraemic subjects compared with 75 controls [801]. This finding is in agreement with a later report which indicated that during haemodialysis, the total serum LDH activity may increase, decrease or remain unchanged [802]; isoenzyme analysis revealed a significant increase during haemodialysis of $LD_5$ in patients with renal tubular disease (acute tubular necrosis and chronic pyelonephritis) as opposed to those where chronic glomerulonephritis was responsible for the renal failure.

After renal infarction, serum LDH rises within one or two days whereas the urine LDH activity rises after 4 days; both elevations persist

for about 10 days [803]. In subjects during rejection of renal transplants, the serum and urine total LDH activities become elevated, and the serum isoenzyme pattern shows a predominance of $LD_4$ and $LD_5$ in contrast to subjects who tolerate the transplant and show the usual predominance of $LD_1$ and $LD_2$ in serum [804]. Less than 6% of 35 patients undergoing transurethral resection of the prostate demonstrated raised serum LDH activity [805].

*Hepatobiliary disease*

Although much historical interest centred on serum LDH in this group of diseases, it is generally regarded as too non-specific to be of value in primary diagnosis [769, 806]. As already mentioned, it may help in the detection of secondary liver damage in primary diseases such as pulmonary or myocardial infarction, especially if a raised $LD_5$ level is sought. The significance of this abnormality as an index of hepatic damage was first described by Wieme and Van Maercke [713]. $LD_5$ elevation is especially pronounced in viral hepatitis and indeed this may occur before the onset of jaundice [807]. However, it is also raised in many other hepatobiliary diseases, including obstructive jaundice, so that its discriminatory capacity in the differential diagnosis of these diseases is poor [772, 807], although it has been reported that the increase in $LD_4$ is greater than that of $LD_5$ in biliary obstruction [694]. If LDH is thus of little diagnostic use, it may nevertheless have some prognostic value in liver disease, as Wieme and Demeulenaere [437] have stated that it is the earliest serum enzyme to return to normal after an episode of viral hepatitis; failure to do so suggests a subacute or chronic course, or even incipient liver failure, while monitoring of $LD_5$ yields even more specific information on these possible complications and seems to be especially useful, according to these authors, in following the course of chronic active hepatitis.

*Other malignant diseases*

As previously mentioned, total serum LDH activity determination is commonly incorporated into multiple sequential analysers. In this setting, it can be a powerful screening test for the detection of patients with malignant disease and in one study seemed superior to 5 other enzymes measured [808]. It has been reported that measurement of total LDH and of $LD_5$ activities are especially useful in the detection of hepatic

metastases and in following the progress of the patient during therapy [809, 810].

Approximately 60% of patients with leukaemia have raised serum LDH activity, $LD_2$ and $LD_3$ or $LD_{2-4}$ predominating, but the patterns are not distinctive enough to allow classification of the type of leukaemia [811, 812]. Although few patients with ovarian tumours have raised total serum LDH, it is more common to find the serum $LD_3$ and $LD_4$ content increased [813]. Elevation of the serum $LD_1$ and $LD_2$ has been reported in patients with teratoma or seminoma of the testis [809].

One of the significant biological facts concerning the relationship of LDH isoenzymes to cancer is the switch from an $LD_1$ to an $LD_5$ pattern in many tumours. This is associated with a higher glycolytic rate in tumours and is consistent with regression to a foetal type of isoenzyme pattern. The importance of this generalisation and an account of the observations on which it is based are fully covered in the monograph by Latner and Skillen [8].

*Anaemia and haemolysis*

Marked elevations of serum LDH activity have been well documented in patients with megaloblastic anaemia [814–816]. These changes are unrelated to the degree of anaemia or the serum levels of folate or vitamin $B_{12}$. The main increase occurs in $LD_1$ and $LD_2$, and there is good evidence from electrophoretic studies that nucleated red cells present in the bone marrow are the source of the increase [817, 818].

Haemolysis of the sample is a common artefactual cause of raised serum LDH activity [819], and this can occur imperceptibly with spurious results when blood samples are transferred by vacuum-tube systems prior to separation of the plasma [820]. This latter situation has its analogy in certain clinical conditions, notably in patients with heart-valve prostheses, where red cells tend to be disrupted during passage through the artificial valve, with consequent elevation of the serum LDH activity [821, 822]. A study of LDH in various forms of haemolytic anaemia has shown that only slight to moderate increases occur in hereditary spherocytosis and sickle cell anaemia, whereas patients with intravascular haemolysis (especially paroxysmal nocturnal haemoglobinuria) had pronounced elevations [823]. This author reported a half-life clearance of 2.7 hours and 135 hours for $LD_1$ in these subjects and showed that these data represented biologically active clearance rather than spontaneous denaturation.

*Muscle disorders*

Normal human skeletal muscle contains a preponderance of M-type LDH isoenzymes. It was of considerable interest that patients with Duchenne muscular dystrophy had relatively increased amounts of $LD_1$ and $LD_2$ in their affected muscles [717, 824, 825]. However, this change is not specific for muscular dystrophy, as patients with many types of disorders, both congenital and acquired, may show decreased muscle content of M-type LDH isoenzymes [826]. Chronic alcoholics also commonly demonstrate a myopathy, and this is associated with reduced content of $LD_5$ and increased content of $LD_1$ and $LD_2$ in their muscles, together with increased $LD_1$ and $LD_2$ in their serum [827]. While of interest for the pathogenesis of muscle diseases, these observations have not led to tests meriting routine application.

## THE AMINOTRANSFERASES (EC 2.6.1.1.; GOT; Aspartate aminotransferase, and EC 2.6.1.2.; GPT; Alanine aminotransferase)

The introduction of these two enzymes into clinical use led to a decisive advance in diagnostic enzymology during the late 1950s. GOT is most abundant in liver, heart and skeletal muscle and exists as a soluble and as a mitochondrial enzyme; the two forms are easily distinguishable by kinetic and electrophoretic criteria [828–831]. GPT is more restricted in its distribution, being found only in the soluble fraction of the cell and in high concentration only in the hepatocyte. Genetic polymorphism of mitochondrial GOT [832] and soluble GPT [833] has been described in human tissues.

### METHODS OF ASSAY

The reaction essentially catalysed by these enzymes is as follows:

$$\text{L-aspartate} + \text{2-oxoglutarate} \overset{\text{GOT}}{\rightleftharpoons} \text{oxaloacetate} + \text{L-glutamate} \quad (1)$$

$$\text{L-alanine} + \text{2-oxoglutarate} \overset{\text{GPT}}{\rightleftharpoons} \text{pyruvate} + \text{L-glutamate} \quad (2)$$

*Colorimetric methods*

The first methods utilised coupling of the pyruvate generated by Equation (2) with dinitrophenylhydrazine to form the brown dinitrophenylhyd-

razone derivatives. Oxaloacetate formed in Equation (1) can be decarboxylated to yield pyruvate; formation of the dinitrophenylhydrazone of pyruvate can thus be employed as the colorimetric end-point for the assay of both enzymes. Extraction of the pyruvate with toluene prior to colour formation [834, 835] is preferable to carrying out the coupling reaction directly on the complete incubation mixture as in the alternative procedures [836, 837]. The simplicity of the latter and their wide availability in commercially prepared kits led to their use in most hospital laboratories, but the poor precision of the methods and their susceptibility to interfering compounds present in serum obscured many important aspects of the relationship between the two enzymes in various forms of hepatobiliary disease [838].

Other colorimetric assays depend upon coupling the oxaloacetate produced in Equation (1) with diazonium salts; they are therefore applicable only to determination of GOT. Fast Ponceau L was first introduced for use with the AutoAnalyser [839]. Although sensitivity increases with increasing pH, the colour reaction should be conducted below pH 4.8, otherwise coupling with 2-oxoglutarate becomes significant [840]. Another diazonium salt used for GOT estimations is Fast Violet B [841, 842]. A comparison has been made of GOT assays using Fast Ponceau L and Fast Violet B, and optimal conditions for both have been defined [843]. As these salts couple with acetoacetate, *p*-aminosalicylate, and even glucose and urea, serum blanks are necessary; for that reason, a third diazonium salt, Fast Red KL, has been advocated and is reported to be more specific for oxaloacetate [844].

Another colorimetric assay for GOT is based upon the oxaloacetate in Equation (1) being coupled to the formation of reduced Coenzyme A (CoA) through the mediation of citrate synthase (CSase) thus:

$$\text{oxaloacetate} + \text{acetyl-}S\text{-CoA} \xrightarrow{\text{CSase}} \text{citrate} + \text{CoA-SH} \qquad (3)$$

The reduced CoA can be detected by its reaction with 5-*S'*-dithiobis-(2-nitrobenzoate) to form a compound absorbing strongly at 412 nm [845, 846]. A variety of modifications to this procedure have been proposed, ranging from a screening test in which the reaction mixture is spotted on to filter paper to a continuous monitoring of the absorbance change.

### *Spectrophotometric methods*

The original procedure for GOT was introduced by Karmen [847] and depended upon coupling the oxaloacetate produced in Equation (1) to

NADH oxidation by means of malate dehydrogenase, thus:

$$\text{oxaloacetate} + \text{NADH} + \text{H}^+ \xrightleftharpoons{\text{MDH}} \text{malate} + \text{NAD}^+ \quad (4)$$

In similar fashion, the pyruvate generated in Equation (2) may be linked to NADH oxidation utilising lactate dehydrogenase [848]:

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \xrightleftharpoons{\text{LDH}} \text{lactate} + \text{NAD}^+ \quad (5)$$

These spectrophotometric assays, in which the reaction is monitored by following the decrease in absorbance at 340 nm, have been 'revised' [849, 850] 'optimised' [45, 51] and 'standardised' [49], but as already indicated on page 7, there is no universal agreement on the best method. From a clinical point of view, there is little to choose between the 'revised' and 'optimised' methods, although both are superior to the 'original' procedures [851]. The reagents have been commercially presented in tablet form and this approach has proved reliable on evaluation [852]. As already mentioned (page 6), these methods are now being performed by automatic reaction rate monitors and parallel fast analysers. They can also be carried out as 2-point or 3-point assays on continuous flow analysers [25, 853], and results with such equipment compare well with those obtained by continuous rate monitoring [853a].

*Technical problems*

The major current problem with aminotransferase assays is the status of pyridoxal phosphate, a cofactor for both enzymes. It was originally believed that both were normally saturated in human serum and that contamination of commercial MDH preparations, used in the spectrophotometric assay of GOT activity, with inactive apoenzyme of GOT could lead to its reactivation by an excess of pyridoxal phosphate present in most sera [854]. It has now been established beyond dispute that aminotransferase activities of many sera can be augmented when the reaction mixture is supplemented with pyridoxal phosphate. Mean augmentation of GOT activity by 16% [855] and 37% [856] has been reported, while GPT activity was stated to be rarely increased beyond 15% in the presence of pyridoxal phosphate [856]. It had earlier been speculated that the low GOT activity found in more than half of a series of patients undergoing long-term haemodialysis was due to loss of pyridoxal phosphate from the serum [857].

The difficulty with pyridoxal phosphate supplementation is that the concentration needed to generate maximal activity varies from one serum

to another. Moreover, it is difficult to ascertain when activation is complete because it is time-dependent as well as dependent upon pyridoxal phosphate concentration; on the other hand, non-enzymatic transamination can be brought about by increasing pyridoxal phosphate concentration, leading to high blank values. There is therefore no widely agreed concensus on the optimal concentration of pyridoxal phosphate to use in such assays, or indeed whether it should be used at all.

Another source of error relates to the interference of serum proteins in GOT assays. Free amino groups of protein accelerate break-down of oxaloacetate and lower results of colorimetric assays with diazonium salts [858]; protein also seems to enhance dialysis of oxaloacetate in autoanalyser systems and can thereby influence apparent GOT activity as measured in such systems. It has been known for some time that when the exogenous commercial enzymes required for spectrophotometric assays are prepared as ammonium sulphate suspensions, erroneous high results may be obtained in sera with raised glutamate dehydrogenase activity (GDH) according to the following reaction:

$$NH_3 + \text{2-oxoglutarate} + NADH + H^+ \overset{GDH}{\rightleftharpoons} \text{L-glutamate} + NAD^+ \quad (6)$$

This source of error has recently been re-emphasised [859], but most commercial enzymes are nowadays prepared in glycerol so that this problem is appreciated and the means of overcoming it are being provided.

## CLINICAL APPLICATIONS

The most important diagnostic uses to which serum aminotransferase estimations are put lie in the fields of myocardial infarction and hepatobiliary diseases. These topics are covered on pages 81 and 88 respectively.

Values of both enzyme activities in serum are raised in drug overdose [860] and even during salicylate therapy [861]. High levels of both are also seen in Duchenne Muscular Dystrophy [862], with abnormal levels being frequent but less pronounced in dermatomyositis and myoglobinuria [863, 864]. In some cases of muscular dystrophy, the mitochondrial as well as the cytoplasmic isoenzyme of GOT may be found in the blood

serum, its presence being related to the severity of the disease [865]. Many cases of alcoholism have raised serum GOT even when no stigmata of liver disease are present; this is now known to be due to a myopathy which not uncommonly complicates this condition [866].

Various lung diseases may be associated with raised serum aminotransferase activities, although the abnormalities are rarely of help in diagnosis. Bronchial carcinoma is one such condition [775, 867]. Elevations are occasionally encountered in pulmonary tuberculosis and do not correlate with activity of the disease process [868]. Findings in pulmonary embolus have been controversial. Early investigators reported no increase in serum GOT in this condition [869, 870] but it was subsequently claimed that elevations are found in approximately 50% of cases [871, 872]; such elevations were stated to be relatively modest and to begin 3–4 days after the embolic episode; it was therefore not too difficult to distinguish these abnormalities from the pattern of GOT increase after myocardial infarction. A more recent study suggested that GOT, which was elevated in the serum of one-third of cases of acute pulmonary embolism, derived from extra-pulmonary sources such as liver and erythrocytes [873].

Urinary GOT activity is raised in the presence of glomerulonephritis [795] or renal tract infection [799], while serum GOT is elevated after renal infarction, the time course resembling myocardial infarction in that peak activity is reached within 24–36 hours and values return to normal after 4 days [803].

Variable changes, or no changes at all, in serum GOT activity are seen in haematologic diseases [863]. This is somewhat surprising, since erythrocytes are well-endowed with this enzyme. Its activity, however, decreases in older erythrocytes due to permanent inactivation, and reversible loss of pyridoxal phosphate from the apoenzyme [874]; measurement of erythrocyte GOT activity provides an index of the proportion of young to old cells in the sample. An important consequence of the high erythrocyte GOT content is the danger of spurious increase of serum activity due to haemolysis of the sample, or leakage of the enzyme from the cells in unseparated samples [819, 875].

About 25% of patients sustaining acute brain haemorrhage demonstrate raised GOT activity, an incidence similar to that occurring with creatine phosphokinase, but the elevations take place later than those typically seen after myocardial infarction [876]. In contrast to the latter enzyme, raised serum GOT activity is uncommon after extensive surgical procedures [877] and cardiac catheterization [878].

## CREATINE PHOSPHOKINASE (EC 2.7.3.2.; CPK; ATP:Creatine phosphotransferase)

The first report outlining a clinical application for this enzyme came from Japan in 1959 [879]. These workers described very high activities in the serum of patients with Duchenne muscular dystrophy – a severe and ultimately fatal disease transmitted as an X-linked recessive disorder and consequently passed on from female carriers to their male offspring. This finding was soon confirmed by French investigators [880] who also extended the clinical utility of the assay by observing raised activities in patients with myocardial infarction [881]. Intensive research over the next decade established this as one of the most important serum enzyme tests in laboratory medicine. An extended account of its behaviour in disease states is therefore necessary.

### TECHNIQUES OF ASSAY

CPK catalyses the following equilibrium reaction:

$$\text{creatine} + \text{ATP} \rightleftharpoons \text{creatine phosphate} + \text{ADP} \qquad (1)$$

The ADP produced may be linked through pyruvate kinase and lactate dehydrogenase, acting as auxiliary and as indicator enzymes respectively, to oxidation of NADH which may be conveniently monitored spectrophotometrically at 340 nm [882]:

$$\text{ADP} + \text{phosphoenolpyruvate} \xrightarrow[\text{kinase}]{\text{pyruvate}} \text{pyruvate} + \text{ATP} \qquad (2)$$

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow[\text{dehydrogenase}]{\text{lactate}} \text{lactate} + \text{NAD}^+ \qquad (3)$$

An alternative fixed-point colorimetric procedure has been presented in which pyruvate generated in Equation (2) is coupled with dinitrophenylhydrazine [883]. An automated method of CPK assay utilises the Equation (1) in this direction and measures the formation of creatine phosphate [884]. A semi-quantitative screening technique has been described in which the time taken for NADH fluorescence to disappear through Equation (3) is measured by spotting an aliquot on filter-paper and examining under UV light [885].

It happens, however, that the equilibrium of Equation (1) lies far to the left and the sensitivity of the enzyme assay can be greatly increased by making use of this fact. Colorimetric methods based upon determination

of free creatine have been described [886–888], and two automated methods utilise this principle [889, 890]. Sensitive assays have become possible through use of a fluorochromatic reaction to measure free creatine [891–893] and two automated versions of this technique have been published [894, 895].

Perhaps the most widely-used method at the present time couples ATP generation from the substrate creatine phosphate through hexokinase as auxiliary enzyme and glucose 6-phosphate dehydrogenase as indicator enzyme, the latter leading to formation of NADPH which can be monitored continuously [896, 897]:

$$\text{ATP} + \text{glucose} \xrightarrow{\text{hexokinase}} \text{glucose 6-phosphate} + \text{ADP} \qquad (4)$$

$$\text{glucose 6-phosphate} + \text{NADP}^+ \xrightarrow[\text{dehydrogenase}]{\text{glucose 6-phosphate}} \text{6-phosphogluconate} + \text{NADPH} + \text{H}^+ \qquad (5)$$

The reagents for this assay are available commercially in the form of tablets or capsules [898, 899], or they may be prepared as a single stable solution [900]. The method has also been modified and adapted for automatic rate analysers [901].

CPK is a magnesium-dependent enzyme. Initial reports claimed that the enzyme was very unstable on storage, but this was shown to be due to oxidation of SH groups and can be prevented by incorporating a thiol compound into the reaction mixture [886, 902, 903]. Dithiothreitol and dithioerythritol are the most useful thiol compounds in this regard, as cysteine and mercaptoethanol are unstable, and glutathione introduces the risk of a competing side-reaction in sera with raised glutathione reductase activity [901, 904]. It has also been stated that albumin has a protective effect on CPK activity [905].

A controversial aspect of serum CPK activity concerns the effect of dilution upon the apparent activity which increases with the degree of dilution [906–910]. Although the phenomenon undoubtedly exists, it is probably a technical artefact and claims that it is specific for muscular dystrophy sera [908, 909] have not been substantiated [907, 910]. Although the present reviewer has obtained some direct evidence for the presence of CPK inhibitors in human serum [888], dogmatic assertions concerning their existence, such as those of Thomson [908, 909] seem unwarranted if based only upon the tenuous data presented by that author. Equal uncertainty surrounds recent claims that dialysis raises serum CPK

activity of subjects with dermatomyositis, lowers activity of subjects with Duchenne muscular dystrophy and has no effect on sera from patients with limb-girdle muscular dystrophy [911,912].

## CLINICAL APPLICATIONS

### *Myocardial infarction*

Early work was carried out with a wide range of methods, many of them technically unsatisfactory, insensitive, and prone to chemical interference. Nevertheless, the consensus gradually emerged that the serum activity of CPK is raised in patients after myocardial infarction more promptly, more extensively, and more specifically than that of other serum enzymes utilised thus far [913–921]. Its role in the evaluation of patients with ischaemic heart disease will be deferred (page 81) since it is best considered in relation to the recommended serum enzyme profile used in such investigations.

### *Muscle disease*

Methodological problems unquestionably coloured some of the earliest reports, but it soon became widely accepted that high values were pathognomonic for Duchenne muscular dystrophy [914,916,920,922–926]. Lesser activities were recorded in subjects with more benign forms of muscular dystrophy such as limb-girdle, facio-scapulo-humoral, and myotonic varieties. The main problem concerns the fact that activity in Duchenne muscular dystrophy is proportional to the total body mass of diseased muscle [924,926,927]. As the diseased muscle atrophies and is replaced by fibrous tissue, the contribution of CPK to the serum pool diminishes and its activity in serum declines. This is useful in following the course of the disease once the diagnosis has been established; but when a patient presents for the first time when the disease is relatively advanced, as occasionally happens in the less-common autosomal recessive variety which follows a more benign course, serum CPK activity may lie at a level which does not permit a distinction to be made between this condition and the other forms of muscular dystrophy.

As a consequence of the studies listed above, it was accepted that in addition to the muscular dystrophies, certain other primary diseases of muscle such as polymyositis may give rise to elevated serum CPK activity; by contrast, it was alleged that elevations were rarely encoun-

tered in neurogenic disease leading to secondary muscle involvement. This concept has required revision in the light of reports drawing attention to the high frequency of moderately elevated serum CPK activities in motor neurone disease [928, 929]. Raised values have also been described in the Kugelberg–Welander syndrome of familial hereditary proximal spinal atrophy [930] and in familial hyperkalemic periodic paralysis [931]; the former disease is primarily neurogenic, and the latter is not associated with any known morphologic abnormality of muscle tissue. Tetanus is another condition which may cause raised serum CPK activity without there being evidence of structural muscle damage at the cellular level [932]. Experimental work has revealed the mechanism as being more probably related to a direct effect of the toxin upon the muscle membrane than to the intense muscular spasm per se [933]. The report of serum CPK elevation in chronic alcoholics during acute alcohol intoxication emphasizes the susceptibility of muscle tissue to secondary toxic damage [934].

In view of the large concentration of CPK in skeletal muscle, it is hardly surprising that the serum level may be profoundly altered by muscular activity. This was conclusively shown by Griffiths [935], although an earlier report in which subjects were tested one hour after exercise had denied such an effect [936]. This disagreement was resolved in a detailed study of the kinetics of CPK release after exercise showing that the peak was not attained until 8–16 hours after its cessation [937]; moreover, the extent of serum CPK elevation for a given level of physical exercise can be reduced by training. A later study compared the effects of exercising normal subjects and patients with motor neurone disease [938]; the authors concluded that in disease states associated with increased permeability of muscle membranes, an exaggerated response to exercise was to be expected, but this became diminished as the total body muscle cell mass was reduced by the disease process. The effect of exercise in health and disease is a factor that must clearly be taken into account in the differential diagnosis of disease states, and even more so in the detection of subclinical disease and genetic carrier status as outlined below. So, too, must the well-established observation that intra-muscular injections may occasion transient and striking increases in serum CPK activity [939, 940].

*Genetic counselling*

Since the Duchenne type of muscular dystrophy follows the inheritance pattern of an X-linked recessive state, half the daughters of a heterozy-

gote female carrier will be normal and half will be heterozygote carriers of the abnormal gene; half the sons will be affected by the disease. Considerable effort has therefore been expended on attempts to define female carriers, since they may prefer not to have children or to limit their offspring to daughters, through therapeutic abortion consequent upon amniocentesis and sexing of the foetus. Many studies exploring the value of serum CPK determinations in this situation have been conducted, with variable results, since the initial reports of Richterich and his associates [941, 942]. Subjects are usually classified as definite carriers (female with one dystrophic son and one or more dystrophic brothers), probable carriers (mother of two or more dystrophic sons), and possible carriers (any relative of a dystrophic patient). Some authors have claimed an incidence of elevated serum CPK activity of greater than 75% in definite carriers [943–947], but others have found a lower incidence [888, 926, 948, 949]. Provocation tests based upon exercising the subject have been proposed as a means of improving the accuracy of carrier diagnosis [950–952], but so far these have not become reliable and established procedures.

Mention should be made of a more benign form of X-linked progressive muscular dystrophy designated the Becker variety. CPK activities in the serum of affected subjects tend to lie well below the levels attained in cases of Duchenne dystrophy. However, female carriers may have elevated levels [943, 952], and an incidence of 50% among definite carriers has been reported [953].

### *Cerebral disease*

Raised serum CPK levels have been recorded in a number of neurologic diseases, including cerebral infarction, meningitis and encephalitis [954, 955]. The elevations tend to occur relatively slowly in relation to the onset of symptoms, in general reach only moderate proportions, and do not seem to be related to the prognosis. Indeed, it has been clearly shown that the source of the elevation is not the damaged tissue and is most probably skeletal muscle [956, 957]. High serum levels of CPK also occur after epileptic seizures and have been shown to be due to the muscular convulsions rather than to activity of an abnormal brain focus [958]. Patients with acute psychosis may also manifest extremely high values for serum CPK, and once again leakage from skeletal muscle seems probable [959].

It thus appears that serum CPK activity cannot even distinguish organic from functional cerebral abnormality, is not primarily related to the abnormality, and is of little use in predicting the course of the illness. Rather than a diagnostic aid, it represents an embarrassment and diminishes the clinical utility of the test in those conditions where it is genuinely valuable.

*Myxoedema*

It is by now well established that serum CPK activity is raised in hypothyroidism [960–963]. The mechanism of this elevation is not fully understood. The CPK isoenzyme pattern of thyroid tissue is different from that of brain, myocardium and skeletal muscle [963]. Griffiths [962] reported that the serum CPK enzyme pattern in patients with myxoedema resembled that of myocardium; it is therefore unlikely that thyroid tissue itself is the source of the elevation. On the other hand, serum activities of CPK and adenylate kinase, which increase in parallel in diseases of both myocardium and skeletal muscle, become dissociated in thyroid disease, since adenylate kinase is elevated in thyrotoxicosis and reduced in myxoedema [964]. This suggested to the authors that if skeletal or cardiac muscle is the source of both enzymes in thyroid disease, their release could not be due simply to diffusion through impaired cell membranes.

The references cited above, and several others mentioned by Wilkinson in his review [965], are at variance in their assessment of the diagnostic usefulness of CPK estimations in hypothyroidism.

Two other causes of raised serum CPK activity are mentioned at this juncture as they may complicate hypothyroidism, and, even when they do not, it is probable that they generate the abnormality by a similar mechanism. One is hypothermia [966]; the other is suicidal drug intoxication with coma [967].

*Malignant hyperpyrexia*

This syndrome was first described in 1960 [968] and manifests itself as a complication of general anaesthesia in which generalized muscular rigidity, severe metabolic acidosis, and a rapid rise in body temperature develop. It is inherited as a Mendelian dominant and is fatal in a high proportion of subjects [969]. Several years ago, it was established that a high proportion of patients and their relatives had raised serum CPK activity [970, 971]. A more detailed study subsequently showed that

subjects in this category did not, on the whole, suffer from a clinically-detectable myopathy, but those propositi and their families who were affected by the condition and had normal CPK activity often had an associated myopathy [972]. In an attempt to define patients at risk, muscle biopsy procedures and in vitro tests of muscle contraction after exposure to halothane and suxamethonium have been developed; these are more accurate than serum CPK assays [973, 974].

The problem of interpretation of CPK assays as screening procedures for such patients stems from the low incidence of the abnormality in the anaesthetized population (ranging from 1 : 14000 to 1 : 75000), and the fact that, after general anaesthesia [975] and indeed in many subjects before elective surgery and for no apparent reason [976], serum CPK levels may be elevated. Situations in which these anomalies may occur have been summarised by King and Zapf [977].

## CREATINE PHOSPHOKINASE ISOENZYMES

It has now been established that there are three principal molecular forms of CPK. Brain and most non-muscle tissues containing detectable activity contain a single band which moves fairly rapidly towards the anode. Skeletal muscle contains predominantly one slow-moving band. Cardiac muscle contains a band of intermediate mobility as well as a slow-moving band corresponding to that of skeletal muscle. Realisation has grown that these bands consist of dimeric forms of the enzyme [978], and they are usually designated as BB, MM and MB respectively. Many techniques have been used to separate CPK isoenzymes. Various support media have been selected, notably cellulose acetate [979–981], starch [978], agar gel [982], agarose [983–985], and poly acrylamide gels [986]. Most visualisation procedures have made use of the coupled system involving monitoring of ATP-production by the hexokinase/glucose 6-phosphate dehydrogenase reactions yielding NADPH, which may be used to reduce a formazan dye [982, 984], or may be detected by fluorescence scanning [985, 986]. Separation of CPK isoenzymes on DEAE-Sephadex columns has also been achieved and can be applied almost on a routine scale [987, 988].

In view of the diversity of techniques employed, it is not surprising that some minor differences in the basic patterns described above have been noted by some authors. Additional bands may be given by myokinase unless its activity is adequately suppressed by AMP [982, 983], and 'nothing dehydrogenase' can also be a problem [985]. For example, Madsen [983] found two extra bands in brain tissue when cysteine was

incorporated in the staining medium. Smith [986] claimed to resolve the MM band into 3 adjacent conformers and MB into two adjacent bands; he also stated that cardiac muscle contains a band nearer to the origin than the MM band, and that BB may be seen in very active extracts of skeletal muscle tissue.

To an extent, these differences are reflected in disparate findings reported from studies of diseased muscle. Initially, no changes were stated to occur in neuromuscular atrophy, polymyositis, and the muscular dystrophies [989]. A later report defined important differences between foetal and adult skeletal muscle, the former having a high concentration of BB-type isoenzymes which reappeared when adult muscle underwent atrophy [990] or became involved in muscular dystrophy [991]. A more recent study of the muscular dystrophies yielded data indicating that skeletal muscle CPK isoenzymes were unaltered, except for a small proportion of early Duchenne cases whose muscles manifested a foetal-like pattern including the BB-isoenzyme [865]. The same authors examined the serum from their cases, and found that the majority of subjects yielded only the MM-isoenzyme, but one-third of those with Duchenne muscular dystrophy contained detectable amounts of MB-isoenzyme as a minor component.

As mentioned previously, the BB-isoenzyme is not present in human serum, even when cerebral disease or injury is responsible for raised CPK activity [956, 957]. Greater interest has been focused on the MB-isoenzyme because it is never present in normal adult serum and is detectable for a period of about 48 hours following myocardial infarction even though the MM-form remains the predominant isoenzyme in such sera [988, 992, 993]. It thus has a diagnostic potential which has yet to be fully developed and applied on a wide scale.

## SERUM ENZYMES IN THE DIAGNOSIS OF MYOCARDIAL INFARCTION

This aspect of diagnostic enzymology represents an area of increasing work-load for every routine hospital laboratory and one of the most fruitful applications of the subject [994, 995]. The principal tests in common use have already been touched on in this review. None is absolutely specific for myocardial damage, and a profile of enzyme determinations becomes obligatory for accurate diagnosis. The most careful attention to clinical detail is also essential if the data are to be correctly interpreted. No single enzyme test or combination of tests,

however expertly performed, will give an absolutely correct diagnosis in every case; the frontier between true infarction and cardiac ischaemia is as difficult to define enzymologically as clinically, and a multiplicity of diagnostic approaches is more likely to succeed than the techniques of one discipline alone.

## CHOICE OF ENZYMES

Elevated levels of aspartate aminotransferase (GOT) accompanied by normal or only moderately raised alanine aminotransferase activity (GPT) were the first enzymological features of myocardial infarction to be recognised [996, 997]. This pattern is not exclusive to myocardial infarction, but it is found in few other conditions associated with chest pain, hypotension and shock. Lactate dehydrogenase (LDH) was also found to be raised after an episode of acute infarction [848] and still enjoys wide use as a diagnostic test in this condition, but its lack of specificity is a serious drawback. This has been partially overcome by the development of techniques for assessing or quantitating the fast-moving LDH isoenzymes, characteristic of but not exclusive to myocardial infarction, as outlined on page 59. As mentioned previously, stabilities to heat and to urea probably offer the most accurate of the technically convenient methods for quantitating $LD_1$ and $LD_2$, but use of oxobutyrate as substrate–the so-called hydroxybutyrate dehydrogenase activity (HBD)–is widely used, and more extensive knowledge of this test than of any alternative procedure has been recorded in the literature. Most specialist enzyme manufacturers also provide kits for its determination. These advantages compensate to some extent for the fact that it is raised in conditions other than myocardial infarction more frequently than was first thought; its use in isolation cannot be recommended, but it is a valuable component of enzyme profiles directed towards the diagnosis of myocardial infarction.

Aldolase [998] and malate dehydrogenase [999] were among the earliest enzyme tests to be employed in this condition, but they have failed to win an established place in most laboratories; they are raised in so many other conditions that their diagnostic value in any one is low. This criticism applies with much less force to creatine phosphokinase (CPK), first reported to be raised after myocardial infarction by French workers [881]. Confirmation soon followed [886, 1000] and the test was initially regarded as highly specific for infarction. This view is no longer tenable, as described on page 76, but its specificity is still higher than that of any other enzyme. From time to time other enzyme tests have been intro-

duced, pyruvate kinase being one such example [1001, 1002], but none has been able to add to the information obtainable from earlier investigative procedures.

CPK must be regarded as the one essential enzyme in the investigation of myocardial infarction, but of itself it is insufficient. The aminotransferases, especially GOT, are useful adjuncts. LDH and/or HBD, especially the latter, are also valuable complementary tests. In the author's laboratory, CPK, HBD, and the aminotransferases are routinely determined on admission and at two further intervals over the next 48 hours in all patients suspected of myocardial infarction. This regime can be recommended as optimal for diagnosis, for prognosis, and for the early detection of complications such as cardiac failure.

## TIME COURSE OF ELEVATED ENZYME ACTIVITY

As illustrated in *Figure 1.9,* it is generally agreed that, in the uncomplicated case, serum enzyme activities increase sharply to a maximum between 24 and 48 hours and thereafter decline to normal values [1003, 1004]. The maximum is reached most rapidly by CPK, which also

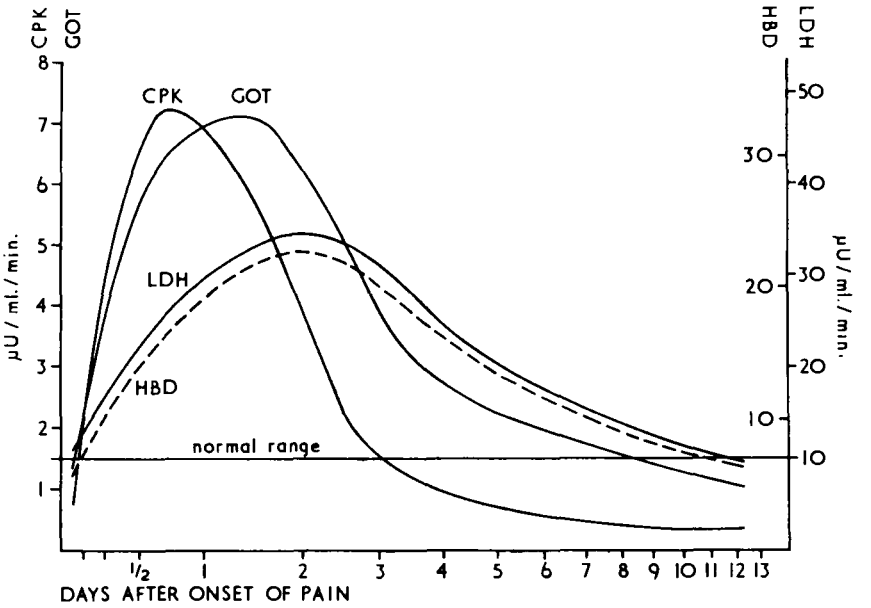


*Figure 1.9. Time-course of serum enzyme elevations after acute myocardial infarction [1003]*

declines most rapidly, this maximum exceeding the normal limit to an extent that is greater than with any other enzyme. The elevation of LDH activity occurs more slowly, is less pronounced, but is of far longer duration, persisting in some cases for more than two weeks. The response shown by GOT and HBD to myocardial infarction is intermediate between the above two patterns, the latter being noteworthy in taking many days to return to normal values.

Should heart failure ensue, the fall in activity of GOT, LDH and, less certainly, HBD may be much delayed, and a steady increase in GPT activity–not uncommonly to values higher than those of GOT–is usually seen. Re-infarction is frequently manifested by a fresh rise in enzyme activity, CPK being especially valuable in this context.

Increased LDH occurs in 30% of patients with heart failure who have not sustained infarction, and the time-course may be indistinguishable from that following infarction [1005].

## DIAGNOSTIC ACCURACY

### *Incidence of raised values*

A summary of some relevant papers detailing experience with GOT, CPK and HBD is presented in *Table 1.6*. Early reports and reviews created the impression that abnormal GOT levels were almost invariably obtained between 12 and 72 hours after myocardial infarction [870, 1019–1021]. Subsequent authors have observed GOT rises less consistently after myocardial infarction, incidences ranging from 80 to 95% being more usual (*Table 1.6*). By the same token, the incidence of raised CPK and HBD activities subsequent to myocardial infarction ranges from 90 to 100% in the series reported by various authors (*Table 1.6*). It is interesting that the experience of those authors who have reported more than one series is not always consistent, emphasizing the influence of case material, numbers, and diagnostic criteria upon the conclusions reached. Enzyme values that are consistently normal, even when samples are obtained between 12 and 72 hours after onset of symptoms, are not in themselves sufficient grounds for discarding a diagnosis of myocardial infarction.

Total LDH activity was reported to be raised in 86% of 282 patients with myocardial infarction in whom the diagnosis was made clinically, and in all of 39 patients in whom autopsy confirmation was available [1022]. This experience seems to be fairly representative [1023].

Table 1.6. ENZYME CHANGES AFTER MYOCARDIAL INFARCTION

| *Reference* | *Total number of cases* | *Percentage with raised enzyme values* | | |
|---|---|---|---|---|
| | | *GOT* | *CPK* | *HBD* |
| 753 | 60 | 87 | – | 100 |
| 1006 | 16 | 70 | – | 100 |
| 1007 | 52 | 98 | 100 | – |
| 1008 | 70 | 100 | – | 100 |
| 1009 | 330 | 78 | – | 100 |
| 1010 | 38 | – | – | 97 |
| 914 | 42 | [a] | 91 | – |
| 1011 | 50 | 92 | 100[b] | 92 |
| 915 | 40 | 100 | 100 | – |
| 918 | 10 | 100 | 100 | – |
| 1012 | 100 | 87 | – | 100 |
| 1003 | 55 | 98 | 89 | 96 |
| 916 | 15 | – | 100 | – |
| 917 | 20 | 100 | 100 | 100 |
| 1004 | 68 | 87 | 96 | 94 |
| 1013 | 28 | 86 | 92 | 93 |
| 1014 | 62 | 100[c] | – | – |
| 1015 | 43 | 98 | 98 | – |
| 919 | 190 | 97 | 100 | 94 |
| 920 | 21 | 72 | 91 | – |
| 898 | 20 | – | 100 | – |
| 1016 | 11 | 91 | – | 91 |
| 921 | 373 | 94 | – | – |
| 1017 | 121 | 93 | 83 | – |
| 1018 | 750 | 92.6[d] | 92.7 | 90.7 |

The values are corrected in so far as possible to exclude cases presenting $< 12$ and $> 72$ h after onset of symptoms.

[a] Stated to be less often raised than CPK but exact data not given.

[b] Based on 21 cases.

[c] Only 76% raised when enzyme measured by alternative method.

[d] Includes cases with higher GPT.

*Role of isoenzymes*

Electrophoretic analysis of LDH isoenzymes, although tedious, frequently demonstrates an increase in $LD_1$ preceding the increase in total LDH [1024], and the diagnostic accuracy of the test is better than 95%. The presence of the MB isoenzyme of CPK is detectable in a high proportion of cases of myocardial infarction, but this abnormality is

transient, and the data so far presented do not point to a clear advantage over total CPK [993, 1025], especially as the MB isoenzyme can be released in carbon monoxide poisoning and malignant hyperthermia [1026]. A recent advance concerns the development of a technique for the immuno-titration of three specific aldolase isoenzymes and the demonstration of a specific pattern in patients with myocardial infarction distinguishable from that found in hepatic and muscle disease [1027], but this approach is in need of further evaluation.

*Incidence of false-positive values*

The lack of specificity of GOT for the diagnosis of myocardial infarction has been well-recognised [863, 1020] and prompted the search for more reliable tests. The many conditions associated with raised total LDH activity have already been documented (pages 64–69), and those responsible for abnormal CPK activity have also been described (pages 76–80). Raised HBD values are often encountered in liver disease, muscle disease, heart failure, and megaloblastic anaemia [1006, 1008, 1028, 1029], and a high proportion of abnormal results occur in pulmonary embolism [1030]. Haemolysis of the sample is also a troublesome source of falsely elevated HBD activity. *Table 1.7* presents an analysis of false positive values for CPK, HBD, and the aminotransferases in 1553 cases suspected of myocardial infarction in whom the diagnosis was ultimately rejected [1018].

In their review, Sobell and Shell [995] compiled data from the literature for the elevation of GOT, CPK and LDH and/or $LD_1$ for the following categories of coronary insufficiency excluding transmural infarction:

(1) Prolonged chest pain with transient ST-segment and T-wave changes (58%).

(2) Prolonged chest pain without electrocardiographic abnormality (27%).

(3) Classical angina pectoris (5%).

*Generalised enzyme elevations*

Not surprisingly, abnormalities are frequent following electrical cardioversion [1031, 1032], open heart surgery [1033], other forms of surgery [805, 1034], and cardiac catheterisation [877]. Hypothermia may also cause striking increases [1035], and so, too, can poisoning [967, 1036] and renal failure [800].

Table 1.7. FINAL DIAGNOSIS IN CASES NEGATIVE FOR MYOCARDIAL INFARCTION SHOWING RAISED SERUM ENZYMES DURING INVESTIGATION [1018]

| | *Number of cases* | | | |
|---|---|---|---|---|
| *Diagnosis* | *GOT raised* | *GOT[a] equivocal* | *CPK raised* | *HBD raised* |
| Lung disease[b] | 22 | 17 | 26 | 29 |
| Congestive cardiac failure | 20 | 24 | 12 | 40 |
| Pulmonary embolus | 5 | 13 | 8 | 18 |
| Cardiac ischaemia | 7 | 2 | 7 | 15 |
| Cardiac arrhythmia | 3 | 8 | 8 | 13 |
| Pericarditis | 4 | – | 5 | 6 |
| Raised from previous episode | – | – | – | 4 |
| Postoperation | 29 | 11 | 28 | 16 |
| Hepatobiliary disease | 2 | 21 | 3 | 9 |
| Cerebrovascular accidents | 16 | 2 | 20 | 15 |
| Epilepsy | 7 | 2 | 7 | 3 |
| Drug overdose | 7 | 1 | 7 | 2 |
| Diabetic coma | – | 1 | 2 | 2 |
| Hypothyroid coma | 2 | – | 3 | 2 |
| Uraemia | – | 3 | 1 | 12 |
| Haemolysis of sample | 2 | – | – | 30 |
| Megaloblastic anaemia | – | – | – | 8 |
| Bacteraemic shock | 3 | 1 | 3 | 3 |
| Others[c] | 8 | 4 | 11 | 11 |
| Total[d] | 128 | 110 | 151 | 238 |

[a] Refers to cases in which both aminotransferases were raised, but GPT > GOT.
[b] Includes asthma and status asthmaticus, chronic bronchitis, and emphysema without heart failure, broncho- and lobar pneumonia, and pulmonary tuberculosis.
[c] Includes cancer, leukaemia and renal disease without uraemia.
[d] Based on analysis of 1553 cases.

### *Enzymes and prognosis*

A relationship between the degree of enzyme elevation, the extent of infarction, and the percentage mortality has been frequently demonstrated, but while this holds in general it may not be true for any one individual subject. Rosalki [1008] obtained mean peak LDH, HBD and GOT activities of 868, 646 and 149 IU/litre in 17 patients succumbing during an episode of infarction compared with corresponding values of

665, 480 and 78 IU/litre among 53 patients who survived. Further data on prognosis taken from this paper are given in *Table 1.8.* Mortality increases dramatically when a 10-fold elevation of enzyme activity is attained [1037]. A correlation has also been reported between the peak GOT and LDH values and the size of the infarct as gauged by morphologic criteria [1038].

## ENZYMES AND DISEASES OF THE LIVER AND BILLARY SYSTEM

### THE AMINOTRANSFERASES AND AMINOTRANSFERASE RATIO

In the mid 1950s, a series of reports established these enzymes as crucial tests in the detection and diagnosis of liver disease [1039, 1040]. Wroblewski, who with his associates contributed to these developments, has ably summarised the consensus that emerged from this pioneer work towards the end of the decade [863, 1020].

Characteristically high values of both aminotransferases are an invariable feature of viral hepatitis, and the peak values encountered in this condition are hardly seen with any other disease, reaching between 10- and 30-times normal at the onset of icterus. The elevation commences in the prodromal phase, up to 10 days before icterus is evident; indeed, many subjects with viraemia and the capability of infecting others never develop icterus although their aminotransferases may be greatly elevated.

Table 1.8. RELATION BETWEEN SERUM ENZYME LEVELS AND MORTALITY FROM MYOCARDIAL INFARCTION [1008]

| *Enzyme activity* | *Number of patients* | *Number of deaths* | *Percentage mortality* |
|---|---|---|---|
| LDH (IU/l) | | | |
| >1000 | 10 | 5 | 50 |
| <1000 | 60 | 12 | 20 |
| HBD (IU/l) | | | |
| >750 | 10 | 5 | 50 |
| <750 | 60 | 12 | 20 |
| GOT (IU/l) | | | |
| >150 | 16 | 6 | 38 |
| <150 | 54 | 11 | 20 |

For this reason, their determination represents a powerful tool in studying the epidemiology of the disease and in its prophylaxis through isolation of infected contacts of known cases [1041–1043]. Very early in the genesis of the disease, GOT activity exceeds that of GPT as it occurs in much higher concentration in liver tissue. Because of the slower clearance of the latter, it accumulates and ultimately exceeds the activity of GOT. This is indeed the position in most cases by the time they are first subjected to clinical examination. This ratio is maintained as the aminotransferases, following their crescendo-like rise, fall equally dramatically towards normal [1044–1046], GPT persisting at abnormal levels for a longer time than GOT. When hepatic necrosis is occasioned by toxins such as carbon tetrachloride, not only are high aminotransferase values encountered within a few hours, but the GOT/GPT ratio usually exceeds unity. This happens as a consequence of the simultaneous effect of the toxin upon all hepatic cells, whereas in viral hepatitis the damage is spread over a much longer period with fewer cells being destroyed at any one time. When, on the other hand, the GOT:GPT ratio exceeds unity in an established icteric case of viral hepatitis and the levels are very high, there is a real possibility of acute yellow atrophy leading to a fatal outcome.

When, during the recovery period, a recrudescence of the disease occurs, the downward trend in the aminotransferases is reversed and a further transient rise occurs. This is useful in monitoring therapy; in fact, bed rest and a light diet is the only effective treatment, and failure of the aminotransferases to rise after ambulation can be regarded as evidence of satisfactory resolution. Persistent elevation of the aminotransferases indicates the presence of 'chronic active hepatitis'. When values are moderate and the GOT/GPT ratio is less than unity, 'chronic persistent hepatitis' is probable and the prognosis is good. When the values are above 200 IU/litre and the ratio is greater than unity, 'chronic aggressive hepatitis' is likely, the prognosis being less favourable, and treatment with steroids and immunosuppressants will almost certainly be required.

The aminotransferases are not helpful in distinguishing Type A from Type B virus hepatitis, nor are they useful in detecting asymptomatic carriers of Australia Antigen [1046a]. The hepatitis due to infectious mononucleosis and to toxic drugs such as *p*-aminosalicylate cannot be distinguished from epidemic or serum hepatitis by enzymological tests alone.

Elevated serum aminotransferase levels occur in most types of hepatobiliary disease, as the tests are an index of biliary regurgitation as well as hepatic cell necrosis. Apart from the various forms of pre-hepatic

hyperbilirubinaemia, the jaundiced patient will almost invariably be found to have raised aminotransferase activities in his serum. When attention is devoted to the degree of elevation on the one hand and the activity ratio of the two aminotransferases on the other, a series of criteria can be formulated which serve as a general guide to the type of liver disease with which one is likely to be concerned, and its most probable mechanism. These points were succinctly summarised by Wroblewski [863, 1020] in reviewing the early work of his group, and in general the earlier criteria have proved valid whenever satisfactory assay methods have been employed [994].

Cirrhosis, both micro- and macro-nodular, is associated with aminotransferase levels that may be normal when jaundice is absent, and only slightly raised in the presence of jaundice. Elevations are rarely more than 3-times normal, and the activity of GOT usually exceeds that of GPT.

Raised aminotransferases occur in nearly all subjects with hepatobiliary obstruction, and the levels usually range from 2- to 8-times the normal level, depending upon the degree of icterus. It is uncommon for the activity of GOT to exceed that of GPT when the obstruction is due to non-malignant disease. In a proportion of cases where the obstruction is due to tumour, GOT activity is the higher of the two, and in those cases manifesting hepatic metastases, the proportion reaches about half.

The usefulness of the GOT/GPT ratio has been placed in doubt by the work of several authors employing unreliable assay techniques [422, 1047] but confirmation of its value has been obtained when accurate and reliable methods have been employed [994, 1048, 1049].

Although the GPT is confined to the supernatant of the hepatocyte, a small proportion of the total GOT of the cell is located within mitochondria. This isoenzyme rarely appears in plasma except in very severe liver cell necrosis, when it is then of ominous prognostic significance [72, 73].

As discussed by Coodley [1050], the major disadvantage of the aminotransferases in the diagnosis of hepatobiliary disease is their lack of specificity. Values outside the normal range are found in many patients with diseases of the heart, or skeletal muscle or of the nervous system, and this fact has stimulated many workers to experiment with alternative tests of possibly greater specificity for hepatobiliary disease.

### ISOCITRATE DEHYDROGENASE (EC 1.1.1.42.; ICDH; D-isocitrate: NADP oxidoreductase (decarboxylating))

This enzyme is somewhat more sensitive than the aminotransferases in the detection of relatively minor episodes of hepatic cell necrosis, such as

those which represent reactions to drugs [1051] or alcohol [1052]. It has been reported to show more striking elevation than the aminotransferases in hepatitis associated with infectious mononucleosis [1053, 1054]. Unlike GOT, ICDH is rarely raised following myocardial infarction unless congestive cardiac failure is present, and elevations are rare in myopathy [1055].

Early reports [1055] suggesting that ICDH was valuable in the differential diagnosis of medical and surgical jaundice were not confirmed in a larger series [1056]. This author found slight to moderate increases in activity in many patients with uncomplicated biliary retention. The increases were unrelated to the cause, severity or duration of the obstruction. Increases were found even more frequently in patients with acute inflammation of the biliary tract and in patients with metastatic involvement of the liver. ICDH in itself is therefore of little value in the differential diagnosis of jaundice. It can be assayed fairly conveniently, however, and a technique for use with an automatic rate monitor has been presented [89]. Its superior specificity for the liver complements the non-specificity of the aminotransferases, and all three enzymes are now routinely determined in many laboratories.

### GLUTAMATE DEHYDROGENASE (EC 1.4.1.2.; GDH; L-glutamate: NAD oxidoreductase (deaminating))

Since GDH is a large molecule and is present solely in the mitochondria of the hepatocyte, whereas the aminotransferases are somewhat smaller in size and are present mainly in the supernatant, it has been a commonly held idea that the GDH activity in serum should reflect a somewhat different pathology from that reflected by the aminotransferases. This expectation does not, however, seem to have been realised in a practically useful form.

Early work [1057] suggested that the enzyme had potential value in the diagnosis of hepatic coma since it was elevated in this condition, whereas values were within the normal range in patients with compensated cirrhosis. Schmidt and Schmidt [1048] regarded GDH as valuable in the differential diagnosis of hepatic and post-hepatic jaundice. Considerably raised GDH values in conjunction with moderate elevations of the aminotransferases were suggestive of obstructive jaundice, whereas gross elevations of the aminotransferases with less pronounced GDH elevations were found in patients with acute hepatitis.

Forster, Filippa and Landolt [1058] considered that the highest GDH activities were found in cases of intermittent extrahepatic obstruction

caused by gall stones. However, less marked GDH elevation occurred in many cases of early complete biliary obstruction caused by tumour or stones, as well as in many cases of acute hepatitis. These authors stated that obstructive and parenchymal jaundice can be differentiated by the value of the expression GDH/GPT × 10. Values above 1.5 were indicative of early obstructive jaundice; values below 1.0 were suggestive of parenchymal jaundice; values close to unity were seen in patients with cholestatic hepatitis. Serum GDH was of no value in long-standing extrahepatic obstruction since the enzyme was seldom elevated in this condition. The authors concluded that GDH was a more specific and sensitive indicator of extrahepatic obstruction than alkaline phosphatase since it responded more rapidly to experimental changes in biliary pressure.

### GUANASE (EC 3.5.4.3; Guanine aminohydrolase)

In an initial study on the measurement of serum guanase in patients with hepatobiliary disease [1059], the authors considered that guanase elevation was indicative of hepatocellular damage; they noted that normal results were found in patients with cirrhosis or simple obstructive jaundice and were of the opinion that the enzyme had potential value in the differential diagnosis of medical and surgical jaundice. Later work from the same laboratory [1060] showed that guanase elevations were relatively greater than GOT elevations in viral hepatitis and that the enzyme was only minimally raised in obstructive jaundice, in contrast to GOT.

Other authors [1050, 1061] supported the view that guanase was useful as a discriminatory test between viral hepatitis and other hepatobiliary disease. They confirmed that characteristically high guanase values were found in patients with hepatitis and that the increase was precocious and of short duration. Although they concluded that guanase was useful in the diagnosis of hepatitis, they failed to indicate in what way it was superior to the aminotransferases for this purpose.

In a major study of 301 patients with hepatobiliary disorders and 99 patients with other diseases, Mandel and Macalincag [1062] confirmed that guanase was elevated in 92% of patients with hepatitis, but noted that 67% of patients with obstructive jaundice and 63% of patients with chronic hepatic disease had elevated values. The enzyme was also raised in 13% of patients whose disease was not hepatic in origin. These authors concluded that guanase was a somewhat less sensitive test of hepatic

injury than GOT but that it deserved more extensive clinical application because of its unresponsiveness to myocardial infarction and to haemolysis.

Renal tissue is rich in guanase and serum guanase activities have been measured in patients with renal disease. While Pradanov and Astrug [1063] concluded that 73% of patients with endemic Balkan nephropathy had significantly raised values, Kebejian and Al-Khalidi [1064] found significantly low values in patients with renal disease. Further work is needed before any conclusions may be drawn concerning the role of guanase in the diagnosis of renal disease and the possible difficulties in employing guanase in the diagnosis of hepatobiliary disease in patients with renal disease.

### ADENOSINE DEAMINASE (EC 3.5.4.4.; ADase; Adenosine aminohydrolase)

Initial observations on the serum activity of this enzyme showed that it was elevated in a very high proportion of patients with cancer and was normal in the majority of non-cancer cases studied [1065]. These authors gave no indication of the clinical stage of the cancer but examination of their data suggests that many cases were pre-terminal and could well have had hepatic metastases.

Koehler and Benz [1066] found that the enzyme was raised not only in cancer but also in viral hepatitis, infectious mononucleosis, miliary tuberculosis and cirrhosis. ADase was considered useful in the diagnosis of late hepatitis since the activity in the serum of patients with acute hepatitis did not return to normal as quickly as the activities of other enzymes [1067, 1068].

In a study of 140 patients, Goldberg, Fletcher and Watts [1049] concluded that ADase was of value in the differential diagnosis of medical and surgical jaundice. Normal ADase was generally found in patients with extrahepatic obstruction due to stone, whereas raised values were found in a high proportion of patients with viral hepatitis or cirrhosis and a proportion of patients with biliary cirrhosis or drug jaundice. Extrahepatic obstruction due to tumour, or with marked inflammation, was sometimes accompanied by raised ADase. Although these observations have been confirmed [1069–1071], the test is not widely performed at the present time despite the fact that convenient spectrophotometric methods suitable for manual or automatic instruments have been presented [90, 1072]. The test has proved to be the most specific single biochemical test for portal cirrhosis in the author's experience.

## OTHER ENZYMES

Elsewhere in this review, the role of alkaline phosphatase, 5'-nucleotidase and γ-glutamyl transpeptidase as indices of biliary obstruction and cholestasis has been described. Another enzyme which may be used in similar fashion is *leucine aminopeptidase.* Initially, elevation of this enzyme activity in serum was thought to be fairly specific for pancreatic carcinoma [1073] but this was subsequently shown not to be so [1074]. It remains none the less a useful alternative to alkaline phosphatase in diagnosing obstructive jaundice although its superiority to the latter has never been demonstrated [416–418, 440, 459, 464]. Raised activity occurs during normal pregnancy [355, 356, 1074a], a fact which limits its use in the diagnosis of hepatobiliary disease in the pregnant female.

Since the human liver contains large amounts of most of the enzymes of intermediary metabolism, as well as a number concerned in metabolic functions special to the liver [1075], it is hardly surprising that almost all enzymes measurable in human serum are prone to manifest high activity in the presence of liver necrosis. *Ornithine transcarbamoylase,* an enzyme of the urea cycle, is present in high concentration in liver tissue and is released very rapidly during liver damage [1076, 1077]. However, serum levels are raised in so many forms of liver disease that the test is of little advantage in distinguishing between them. It is frequently normal in cirrhosis [1049]. On the other hand, elevations are common after anaesthesia, early rises being associated with reduction in blood pressure and late rises with increased post-operative protein catabolism [1078]. *Sorbitol dehydrogenase* is an enzyme absent from normal serum but appearing in the course of hepatic necrosis and active with a wide range of polyol substrates [1079]. It is a less sensitive but more specific index of parenchymal liver injury than other commonly used tests; being elevated in only 44% of cirrhotic patients [1080], it does not provide satisfactory discrimination between medical and surgical jaundice. This also seems to be true of *argininosuccinate lyase* [1081] and *xanthine oxidase* [1082]. Serum *phosphohexose isomerase* activity has been advocated as a valuable test in the diagnosis of acute hepatic damage [1083], but elevations also occur in a high proportion of patients with carcinoma before hepatic involvement is evident [1084, 1085] and the determination now appears to have little diagnostic use [1086].

In contrast to the emphasis laid upon enzyme elevations in liver

disease, mention should be made of the fact that pseudocholinesterase, normally synthesised by the liver and secreted into the plasma in fairly high concentration, falls in parenchymal liver disease with consequent reduction of serum activity; this tends not to happen in extra-hepatic obstruction, so that the test has some diagnostic value [1087]. A somewhat analogous situation is seen with hepatic bilirubin UDP-glucuronyl transferase in Gilbert's disease, where low activities detectable in hepatic tissue biopsies are diagnostic of this congenital condition [1088].

## ENZYMES AND PANCREATIC FUNCTION

The exocrine pancreas elaborates a host of enzymes concerned in the digestion of macromolecular foodstuffs. These include amylase, lipase, and a number of different enzymes responsible for the hydrolysis of dietary protein of which trypsin and chymotrypsin have been most extensively utilised in clinical diagnosis [1089]. The latter are secreted as inactive zymogen precursors. In the lumen of the intestine, into which the enzymes are secreted via the pancreatic duct, trypsinogen is converted to the active enzyme trypsin by the action of enterokinase, an intestinal enzyme which cleaves a terminal hexapeptide from the precursor [1090]. Trypsin, in turn, converts chymotrypsinogen to chymotrypsin and is indeed responsible for activating most other proteolytic enzymes of the pancreas. These act preferentially upon specific peptide links [1091] and their integrated action achieves the complete hydrolysis of dietary protein to its constituent amino acids (*Figure 1.10*). At the same time, this specificity makes it possible to measure the activity of the various enzymes in biological material utilising model peptide, amide or ester substrates containing the preferred bond [1092–1095].

Pancreatic secretion is controlled by a complex system of nervous stimuli, chiefly mediated by the vagus nerve [1096], and by the liberation of hormones from the small intestine [1097, 1098]. The principal enterohormones active on the pancreas are secretin, which stimulates secretion of water and bicarbonate by the centro-acinar and ductule cells, and cholecystokinin-pancreozymin (CCK-PZ) which stimulates contraction of the gall-bladder and release of enzymes by the acinar cells [1099, 1100]. These hormones are liberated upon entry of acid and foodstuffs into the duodenum [1101, 1102].

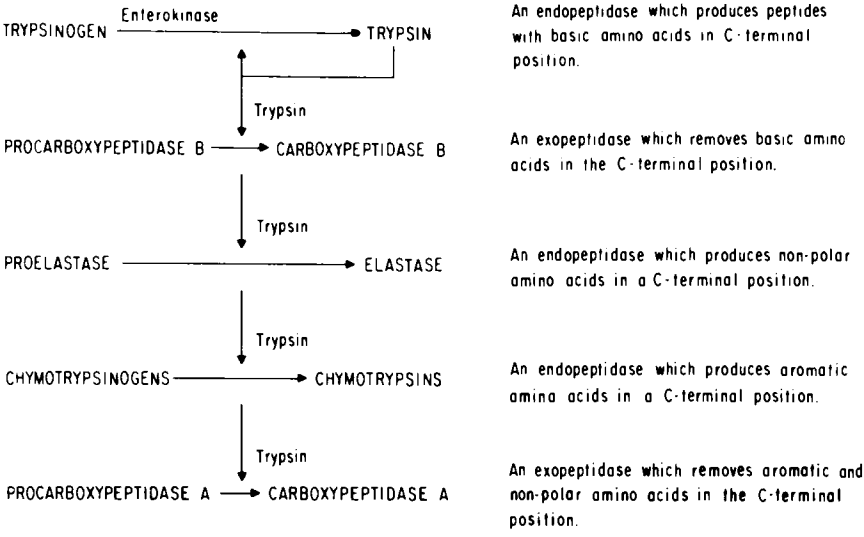


*Figure 1.10. Activation and specificity of pancreatic proteolytic zymogens and enzymes to illustrate integration of enzymatic break-down of dietary protein [1091]*

## TECHNICAL CONSIDERATIONS

### *Duodenal intubation*

To test the function of the exocrine pancreas, it is necessary to collect the secretions of the pancreas after a period of stimulation, since the pancreas does not normally secrete in the resting state. In the test devised by Lundh [1103], this represents no problem, since it is only the concentration of enzyme in the duodenal aspirate (essentially a mixture of bile, duodenal juice, saliva, gastric juice and pancreatic juice) which is measured. More accurate tests rely upon complete collection of the duodenal aspirate so that the output of enzymes per unit of time can be determined. This aim requires separate collection of gastric juice, since regurgitation of pancreatic juice into the stomach is a common occurrence, and gastric juice rapidly destroys the pancreatic enzymes [1104]; prompt neutralisation and refrigeration of the gastric juice prevents this happening. Essentially, therefore, a tube is passed into the stomach and a second into the duodenum to allow the gastric and pancreatic juices to be independently collected. Ideally, some measure of the completeness of

collection is desirable. This can be achieved by utilising a double-lumen tube for the duodenal collection and infusing a marker substance some distance above the point of collection. Polyethylene glycol [1105] and radioactive vitamin $B_{12}$ [1106] have been used for this purpose. Strict fluoroscopic control over the positioning of both tubes is necessary. Other aspects of this technique are described in appropriate reviews [1089, 1107–1109].

*Choice of stimulant*

The pancreas is generally stimulated by secretin alone, CCK-PZ alone, or both hormones simultaneously. Secretin may be given as a single intravenous injection [1110, 1111], by continuous intravenous infusion [1112–1114] or by subcutaneous administration [1115]. CCK-PZ may be given as a single intravenous injection at varying times before or after secretin, or simultaneously with the latter [1111, 1116–1119]. More reproducible results are however obtained when CCK-PZ is given by continuous intravenous infusion [1120], especially when the infusion includes a background dose of secretin [1114]. The enzyme response to intravenous injections of hormones in the doses generally used is submaximal, whereas the maximal secretory capacity of the pancreas is approached during the response to continuous intravenous infusion of hormones.

This is evident from *Figure 1.11* which demonstrates a greater output of trypsin and chymotrypsin with CCK-PZ than with secretin [1121]. Moreover, with the former hormone, output increases with dose-rate up to 8 Crick Harper Raper Units (CHRU)/kg/h; although a slight increase in the mean was observed on doubling this dose-rate, the effect was not significant. It is probable that the maximal secretory capacity of the pancreas is attained at a dose-rate of 16 CHRU/kg/h and approached at half this dose-rate. As shown in *Figure 1.11*, the response to duodenal acidification lay between that provoked by the two doses of secretin.

Indirect methods of stimulating the pancreas have also been used by introducing food materials into the small intestine, thus simulating the pancreatic response to a meal. The most widely used technique of this type at the present time involves the administration of a meal containing carbohydrate, protein, and fat. This was first devised by Lundh [1103] and has been utilised with much enthusiasm and little modification by many other workers [1122–1125]. Under these circumstances, the duodenal contents can be sampled but cannot be quantitatively collected. It is

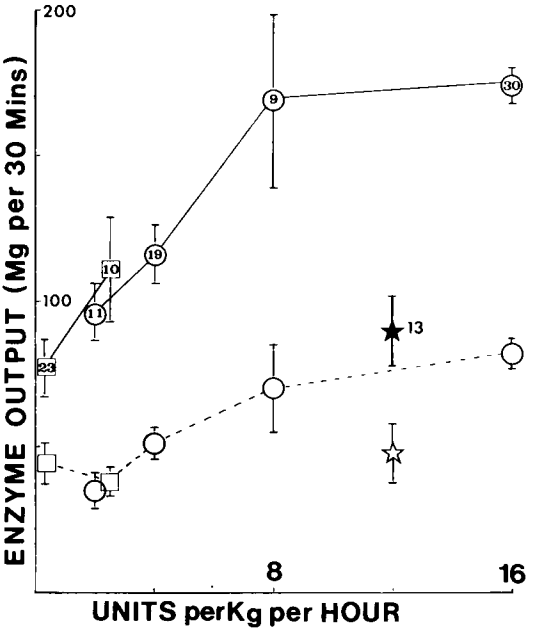


*Figure 1.11. Dose–response relationship of entero-hormones and pancreatic enzyme output in duodenal aspirate. Solid line (trypsin) and broken line (chymotrypsin); squares (secretin) and circles (cholecystokininpancreozymin). Secretin (0.25 clinical units/kg/h) given as background stimulation with all doses of cholecystokinin-pancreozymin. Number of subjects for each regimen given with trypsin results. Points represent mean (±S.E.) for group [1121]. Solid and open stars represent trypsin and chymotrypsin respectively in response to duodenal acidification. Units on abscissa are clinical units for secretin and Crick–Harper–Raper units for cholecystokinin-pancreozymin given by continuous intravenous infusion*

possible therefore to measure only the concentration of enzymes, not their total output; a recent variation of the test utilises incorporation of radioselenium into pancreatic proteins and measures the concentration of this isotope in the duodenal aspirate [1126].

This type of test depends upon the functional state of the small intestinal mucosa and its ability to release the appropriate hormones. The results, being concentration values, are also dependent on the degree of dilution of the pancreatic secretions with those of the biliary tree and small intestine, and even more so on the rate of gastric emptying and flux of the meal through duodenum to small intestine. The deficient pancreatic response to the test meal demonstrated by some patients with duodenal

ulcer [1127] and gastric achylia [1128] doubtless stems in part from these considerations.

Other indirect stimulants include the perfusion of acid [1129], bile acids [1130] and amino acids [1131] into the small intestine, and although these procedures evoke a pancreatic response qualitatively similar to that seen with combined secretin and CCK-PZ stimulation, they not infrequently yield abnormal results in patients capable of responding normally to direct stimulation with the hormones [1109].

### *Normal values and expression of data*

A comparison of the outputs of trypsin and chymotrypsin in response to various stimulant schedules as published by various authors is presented in *Table 1.9.* A problem of considerable magnitude is the difficulty of defining normal limits of enzyme secretory capacity, as the distribution of values for enzyme output in control populations shows marked skewing [1119, 1135, 1136]. A variety of statistical approaches have been used to treat the data so obtained. Normal limits have been calculated assuming a Gaussian distribution [1111, 1117], after logarithmic transformation [1119, 1135], or by defining the lower normal limit as the lowest value obtained in a subject with a proven normal pancreas [1137]. A further controversy surrounds the claim that discrimination between normal and abnormal can be improved by expressing the response in relation to the body weight of the subjects [1122, 1138], since this has been disputed by other authors [1111, 1113].

## DIAGNOSTIC ACCURACY OF PANCREATIC FUNCTION TESTS

Of all the tests used to study pancreatic function, that of Lundh [1103] has been most utilised; this author stated a preference for trypsin estimation because of its superior stability to other pancreatic enzymes in duodenal aspirate, and most other authors have adhered to this preference [1124–1126, 1139] with apparently satisfactory results. The test is most useful in providing evidence of gross pancreatic disease in subjects with steatorrhoea or obstructive jaundice; it is less reliable in patients with lesser degrees of pancreatic functional impairment and is not accurate in distinguishing subjects with chronic pancreatitis from those with other forms of abdominal pain when steatorrhoea is absent [1140, 1141]. The most frequent causes of spuriously abnormal results are diabetes, extrahepatic obstruction, and intestinal villous atrophy [1142]. Some authors

Table 1.9. CONTROL VALUES FOR TRYPSIN AND CHYMOTRYPSIN OUTPUT IN DUODENAL ASPIRATE

| *Reference* | *Control subjects* | *Stimulation* | *Source* | *Trypsin output (μg/kg/min)* | | *Chymotrypsin output (μg/kg/min)* | |
|---|---|---|---|---|---|---|---|
| | | | | *Mean* | *Lower limit* | *Mean* | *Lower limit* |
| 1118 | 18 Healthy male volunteers | Secretin (90 units) and pancreozymin (100 units) | Boots | 25.9 | 11.9 | 18.3 | 10.5 |
| 1122 | 24 Healthy volunteers | Pancreozymin (Cecekin) (3 clinical units/kg) | Vitrum | 23.5 | 4.75 | – | – |
| 1122 | 23 Hospital patients | Pancreozymin (Cecekin) (3 clinical units/kg) | Vitrum | 12.6 | 4.75 | – | – |
| 1132 | 20 Hospital patients free of GI disease | Pancreozymin (Cecekin) (75 Ivy units) and Secretin (1 clinical unit/kg) | Vitrum | 10.9 | 5.6 | – | – |
| 1133 | 7 Normal children | Pancreozymin (1.5 units/kg) | Boots | 11.5 | 7.9 | 12.8 | 6.0 |
| 1134 | 27 Children without pancreatic disease | Pancreozymin (2 units/kg) and secretin (2 units/kg) | Boots | 15.8 | 4.3 | 17.2 | 4.6 |
| 1119 | 123 Patients free of GI disease | Secretin (75 units) and pancreozymin (Cecekin) (75 units) | Vitrum | 26.0 | 9.4 | 19.3 | 7.5 |
| 1121 | 49 Patients with normal pancreatic function | Secretin (0.25 clinical units/kg) and CCK-pancreozymin (4 CHRU/kg) | GIH laboratory | 82.8 | 47.6 | 39.0 | 19.0 |

who have compared the diagnostic efficiency of the test meal with that of hormone tests given in submaximal doses prefer the former [1122, 1143], but others prefer the latter [1144].

Studies utilising hormonal stimulation have tended to include a range of enzymes. This is a wise precaution, since there is increasing evidence that, contrary to previously accepted ideas, the pancreatic enzymes are not secreted 'in parallel' but can manifest dissociation in their response to hormonal stimulation in health and disease [1136, 1145–1148]. There is no agreement as to which single enzyme or combination of enzymes yields the best diagnostic discrimination. In some respects, it appears that one particular enzyme may be most suitable in one particular disease. A number of authors have found the proteolytic enzymes superior to amylase or lipase [1118, 1122, 1132, 1146]. Chymotrypsin was considered superior to trypsin in the diagnosis of pancreatic carcinoma [1149] and kwashiorkor [1133], whereas trypsin was impaired more frequently than chymotrypsin in cystic fibrosis [1134]. A general preference for lipase in pancreatic cancer and chronic pancreatitis has been stated by other workers [1148]. Amylase is of limited value in infancy, as it is low or absent in the pancreatic juice of infants under one year [1150]. As isolated deficiency of trypsinogen may occur [1151, 1152], as well as deficiency of enterokinase in the small intestinal mucosa [1153], it is advisable not to restrict the enzymological investigation of the duodenal aspirate to the proteases.

A satisfactory degree of diagnostic discrimination can be achieved by measuring the output of trypsin and chymotrypsin during stimulation with 4 CHRU of CCK-PZ/kg/h, even when the patients with pancreatic disease have neither icterus or steatorrhoea [1121]. Patients giving abnormal or equivocal results may be tested with 16 CHRU of CCK-PZ/kg/h, and the original collection analysed for amylase or lipase, but occasionally adverse reactions occur at this dosage which is why the lower dose is recommended for initial testing. Although pancreatic function tests of this kind are powerful diagnostic procedures, they are only one of several useful approaches. Best results are obtained when intubation tests are combined with pancreatic scanning and cytology of the duodenal aspirate [1154, 1155].

## FAECAL ENZYMES

To avoid the inconvenience of intubation in the above procedures, it has been suggested that estimation of trypsin and chymotrypsin in the faeces

may give useful information concerning the functional state of the pancreas [1156]. Random samples of faeces [1119] as well as 24-hour collections [1157] have been used, and it is widely agreed that chymotrypsin is much more reliable than trypsin. However, abnormally low chymotrypsin excretion is not infrequent in apparently normal subjects, while occasionally normal values are given by patients with pancreatic disease [1158, 1159]. Normal levels are rare in the presence of pancreatic insufficiency severe enough to cause steatorrhoea so that the test is a useful screening procedure in this situation [1157, 1160].

The efficiency of stool enzyme tests has been greatly improved by carrying out collections after hormonal stimulation of pancreatic secretion [1161]. Good correlation between pancreatic and faecal output, especially for chymotrypsin, was obtained. Use of faecal nitrogen as a reference parameter results in better discrimination than is provided by measurement of the enzymes as an output or in relation to stool weight [1162] as shown in *Figure 1.12.*

## ENZYMES IN THE DIAGNOSIS OF UTERINE CANCER

The accessibility of the female reproductive tract to direct examination and the fact that its secretions drain to the exterior combine to make it highly suitable for the application of enzyme procedures. A powerful stimulus to the development of new diagnostic tests in this field is provided by the high incidence of cancer of the uterus–one of the two commonest malignancies in the female.

### TISSUE ENZYME LEVELS

Direct measurements of enzyme levels in uterine cancer have been relatively sparse. Histochemical techniques demonstrated increase in β-glucuronidase and α-naphthyl esterase in samples of cancer tissue taken from the cervix and corpus of the uterus [1163, 1164]. A more complex histochemical study restricted to the uterine cervix and concerning 13 enzymes revealed variable changes in most dehydrogenase activities, but alkaline phosphatase, 5′-nucleotidase, ATPase and glucose 6-phosphate dehydrogenase were increased in neoplasms, especially those showing the greatest morphologic de-differentiation [1165].

It has been established by conventional biochemical techniques that β-glucuronidase activity is increased in cervical cancer tissue

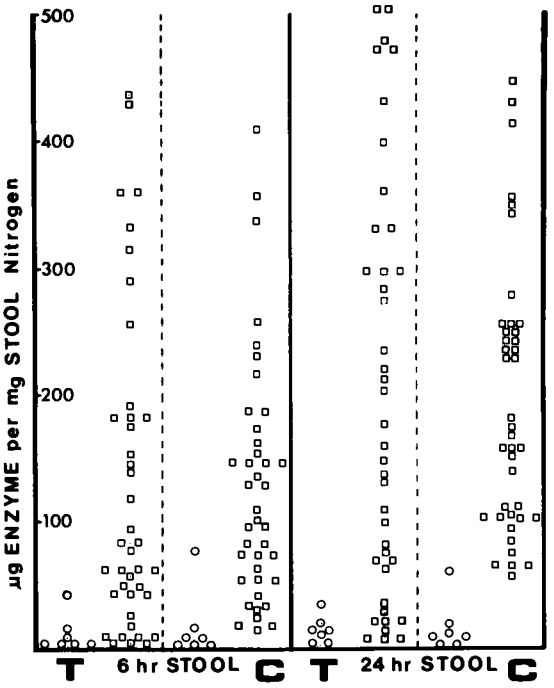


*Figure 1.12 Concentration of trypsin (T) and chymotrypsin (C) relative to nitrogen in 6-h and 24-h stools after stimulation with 1 clinical unit of secretin and 4 Crick–Harper-Raper units cholecystokinin-pancreozymin per kg over a 50-min period. Squares represent subjects with normal pancreatic function; circles represent patients with abnormal pancreatic function [1162]*

[1166, 1167]. Most of this increase occurs in the soluble components of the cell [1168]. Increased activity of 6-phosphogluconate dehydrogenase has also been shown by histochemical and biochemical procedures to be present in cervical cancers [1169–1171] although some authors have been unable to substantiate these findings [1172, 1173]. Several reports claim greatly increased lactate dehydrogenase activity of cervical cancer compared with normal cervix [1172, 1174, 1175] whereas other authors have been unable to find any significant difference [1171, 1176]. However, there is fairly general agreement that there is a preponderance of the slow-moving lactate dehydrogenase isoenzymes in the cancer tissue [1172, 1175, 1177, 1178]. Most other enzymes of glycolysis are increased in cancer tissue [1176], whereas most of the respiratory enzymes are

unchanged [1179]. Very striking increases were observed for adenosine deaminase, deoxyribonucleases and ribonucleases in cervical cancer, the latter being more than 10-fold elevated compared with non-malignant human cervix [1180].

## VAGINAL FLUID

### *Technical factors*

The main diagnostic effort has been directed towards utilisation of material obtainable from the vaginal canal. This may take the form of fluid aspirated from the vaginal fornices – a technique used by the majority of workers in this field, but alternatively the cervical mucous may be aspirated [1181, 1182], or an irrigation procedure may be used to maximise the yield [1183, 1184]. Most authors have related enzyme activity to the volume or to the weight of the sample, either wet weight or the weight after freeze-drying. Activity has also been related to protein concentration [1184–1187], to DNA [1188, 1189], to specific gravity [1190], and to potassium content of the sample [1189].

A variety of preparative procedures have been used prior to enzyme assay, although most workers carry out assays on the untreated sample, or rely upon freeze-drying to liberate intracellular enzymes. Homogenisation with [1185, 1186] and without detergent [1184, 1189] has been used, and in one study the material was filtered in order to determine enzyme activity of the extracellular vaginal fluid [1183]. Ultrasound has also been employed as a means of disrupting the cells [1190]. A critical examination was conducted of the conditions leading to optimum discrimination between malignant and non-malignant vaginal fluid samples and it was shown that separation of cells from extracellular plasma could improve diagnosis [1191]; moreover, the decision whether to use detergent, ultrasound, or neither in the homogenisation procedure, and the choice between carrying out the assay on the whole homogenate or on a high-speed supernate were other factors that can profoundly affect the accuracy of certain enzyme tests. The presence of blood reduces 6PGDH activity of the vaginal fluid [1191, 1192], and it has been reported that there is wide variation in the pH optimum for vaginal fluid 6PGDH activity [1193]. These criteria have received scant consideration from most workers. Failure to do so must be blamed for many of the disappointing experiences reported by some investigators and has contributed to the discordant results obtained by different authors using

variations in technique which, superficially may appear slight, but in reality are capable of exercising a dominant influence on the significance of the data.

*Diagnostic efficiency*

Two enzymes – β-glucuronidase (β-D-glucuronide glucuronohydrolase EC 3.2.1.31) and 6-phosphogluconate dehydrogenase (PGDH, 6-phospho D-gluconate: NAD(P) oxidoreductase EC 1.1.1.44) – have been utilised more extensively than any others. The large corpus of experience with these determinations as isolated procedures is summarised in *Table 1.10*, which is extended from the review by Muir [1194]. A distinction is made, where possible, between invasive carcinoma and carcinoma in situ, the former being implied when the carcinoma is unqualified. Cytological examination of cervical smears has usually been employed as the diagnostic procedure with which the enzyme tests have been compared. Data for carcinoma of the uterine body and other gynaecologic tumours have not been included since, in the experience of most authors, results are uniformly less reliable than those relating to carcinoma of the uterine cervix.

It is immediately apparent from *Table 1.10* that 6PGDH is superior to β-glucuronidase in the detection of patients with cervical cancer, and that neither enzyme is of value in the diagnosis of carcinoma in situ. The incidence of false-positive results in control subjects is variable, but appears to be somewhat higher for 6PGDH. This variability stems from the different clinical material comprising the control groups assembled by various authors. Some have used genuinely healthy populations from screening clinics. Others have used patients with non-malignant gynaecologic disease. These two clinical populations show marked differences in vaginal fluid enzyme activity. Hoffman and Merritt [1205] reported no single incidence of raised 6PGDH activity among 596 pre-menopausal, 219 post-menopausal and 95 pregnant females free of gynaecologic illness; of 1644 patients with benign gynaecologic lesions, 633 yielded high values. The incidence of false-positive values has been reported to be much greater in post-menopausal subjects. Cameron and Husain found an incidence of 29% at age 20–25; 38% at age 40–45; and 80% at age 65–70 [1204]. Age distribution of the population examined is thus a powerful determinant of the false-positive rate for enzyme tests, a fact confirmed for 6PGDH by Muir and Canti [1211], and previously demonstrated for β-glucuronidase by Fishman [1167, 1212, 1213].

Table 1.10. SUMMARY OF EXPERIENCE WITH VAGINAL FLUID β-GLUCURONIDASE AND PGDH IN DIAGNOSIS OF CERVICAL CANCER

| *Reference* | *Carcinoma of cervix*[a] | | *Controls* | |
|---|---|---|---|---|
| | *Total number* | *Percent false-negative* | *Total number* | *Percent false-positive* |
| *Vaginal fluid* | *β-glucuronidase* | | | |
| 1195 | 31 | 0 | 292 | 18 |
| 1196 | 51 | 55 | 500 | 20 |
| 1197 | 44 (2) | 20 (0) | 177 | 20 |
| 1198 | 50 | 52 | 100 | 12 |
| 1181 | 21 | 5 | 85 | 4 |
| 1186 | 112 (5) | 22 (20) | 119 | 40 |
| 1199 | 52 (9) | 27 | 33 | 18 |
| *PGDH* | | | | |
| 1200 | 46 | 0 | 93 | 3 |
| 1201 | 90 (11) | 0 (0) | 1560 | 11 |
| 1202 | – (3) | – (67) | 187 | 19 |
| 1203 | 37 | 37 | 106 | 4 |
| 1204 | 40 (11) | 0 (40) | 2315 | 37 |
| 1205 | 11 (33) | 18 (64) | 2554 | 25 |
| 1206 | 31 (20) | 3 (15) | 108 | 27 |
| 1207 | 6 (7) | 17 (86) | 404 | 30 |
| 1168 | 25 (14) | 0 (16) | 141 | 24 |
| 1186 | 49 (5) | 10 (20) | 64 | 47 |
| 1208 | 13 (33) | 7 (43) | – | – |
| 1209 | 7 (12) | 14 (75) | 2297 | 40 |
| 1190 | 9 (3) | 0 (67) | 731 | 20 |
| 1187 | 157 (66) | 3 (29) | 227 | 56 |
| 1182[b] | 10 (7) | 0 (43) | 476 | 23 |
| 1182[c] | 10 (7) | 20 (57) | 476 | 19 |
| 1210 | 4 (27) | 25 (70) | 3439 | 16 |
| 1183 | 25 | 8 | 30 | 17 |
| 1194 | 69 (18) | 7 (39) | 791 | 27 |
| 1199 | 56 (9) | 9 (67) | 39 | 18 |

[a] Open data refer to invasive cancer; data in parentheses refer to carcinoma in situ.
[b] Utilising cervical mucus.
[c] Utilising vaginal aspirate.

Another source of variability lies in the different normal limits chosen by authors using identical techniques, quite apart from minor differences in technique and attention to interfering factors already described.

Other enzyme tests which have been applied to the diagnosis of cervical cancer include α-mannosidase [1214], phosphohexose isomerase [1215] and ribonuclease [1184]. Results with the former two were not impressive; the latter enzyme appeared more promising. Several papers describe comparative evaluations of various tests carried out in parallel. Lawson and Watkins [1186] found 6PGDH to yield more true positive but also more false positive results than β-glucuronidase. Muir [1215] found 6PGDH to be the most reliable of five enzymes he tested in vaginal fluid of cancer patients. The third paper involved a comparison of β-glucuronidase, 6PGDH and ribonuclease in various fractions of vaginal fluid [1199]. Results with the former were unsatisfactory; 6PGDH and ribonuclease were at least as reliable as cytology in detecting the presence of invasive carcinoma but were ineffective in diagnosing in situ carcinoma. The efficiency of electronic particle sizing, vaginal fluid 6PGDH assay and cytology were compared as screening procedures for cervical cancer; nine cases were detected by cytology, eight by PGDH assay, and only five by particle sizing [1216].

*After cancer therapy*

It has been reported that 6PGDH activity of vaginal fluid increases after radiotherapy [1211, 1217] and that four out of six cancer patients manifested raised activities when free of malignancy after successful therapy [1207]. Operation and radiation often increased the enzyme content of the vaginal fluid, even when the vault was sealed off from the cervix, a situation attributed to changes in the metabolic interaction between host and neoplasm [1186]. Other possible explanations are an increase in the ratio of cancer to normal epithelium in the exfoliated cell population of the vaginal fluid, and escape of enzyme across cell membranes damaged by radiation. This would accord with the observed fall in 6PGDH content of the tumour itself after radiotherapy [1218].

Several reports describe a reduction in vaginal fluid β-glucuronidase content after radiotherapy [1181, 1195, 1198, 1217, 1219]. This is less likely to occur in pre-menopausal subjects [1196, 1217]. Lawson [1197] found an increased β-glucuronidase after treatment in patients showing a good response, but this included hysterectomy and ovariectomy in addition to radiotherapy. A fall in the β-glucuronidase content of the tumour follows radiotherapy [1220].

Activity of ribonuclease was significantly increased, following radiation, in all fractions of the tumour tissue [1218] and in all fractions of the vaginal fluid [1217]. This was subsequently shown to be due to an increased concentration of enzyme rather than to a decreased content of ribonuclease-inhibitor, and the extent of the elevation in the tumour tissue correlated with the response and survival of the patient [1221].

*Current status of vaginal enzyme tests*

After initial excitement, these tests have now fallen into disrepute. The oblivion to which they have been condemned stems from a misconception as to their purpose and misunderstanding about the relationship of in situ and invasive carcinoma of the cervix. There now seems considerable doubt whether many in situ carcinomas do in fact become invasive [1222], and those that do take many years to complete the transition. In this light, the poor contribution of vaginal enzyme tests to the diagnosis of in situ carcinoma becomes less of a handicap. The superior performance of cytology in detecting in situ carcinoma led to the abandonment of enzyme tests, and distracted attention from the fact that the false negative rate for cytology could be higher than that of enzymology in diagnosing invasive cervical cancer [1199, 1206]. Indeed, the false negative rate for cytological screening has been reported to range between 19 and 30% [1223–1225]. The technical demands of cytology are very high, the throughput is very low, and automation of the time-consuming microscopy is in its infancy. A simple test, such as that of PGDH activity in vaginal fluid plasma [1183] can, with modern instrumentation, be performed with such speed and economy that 20 instruments staffed by 40 unqualified personnel could screen the entire population at risk in the United Kingdom annually. Such a program could be complemented by 5-yearly cytological screening to detect in situ lesions. The problem of what to do about the frequent false positive subjects is hardly significant; most of these subjects will be found on further investigation to have benign lesions which would still benefit from medical attention, and the enzyme test would be performing a valuable service in securing therapy for their condition earlier than would otherwise be possible.

## ENZYMES IN GENETIC DISORDERS

Quantitatively, genetic enzyme deficiencies account for the greatest potential application of enzyme determinations to clinical problems – at

least in the sense that more than 100 diseases are now recognised as due to such deficiencies and their diagnosis ultimately depends on demonstrating the deficiency in suitable material. On the other hand, while the range of such disorders is infinitely varied, their incidence is extremely rare, and many medical practitioners will not encounter one such case in their professional life-time. The onus in securing effective diagnosis thus rests upon the specialist who is more aware of their existence and exposed to some tenuous contact with the various syndromes that may arise; help from the laboratory is, to him, a prerequisite in the discharge of this function, but the enzyme assays concerned are so complex that for the most part they can only be offered by a small number of laboratories dedicated to providing this service. All that can be done in this section is to acquaint the reader with the scope of the problem. A number of textbooks provide valuable and authoritative accounts of the role of enzyme assays in hereditary disease [1226–1228].

## GENERAL MECHANISMS

These rare metabolic disorders are due to congenital deficiency of a single enzyme brought about by presence of an abnormal or mutant gene. The inheritance of most such conditions follows a Mendelian recessive pattern. Affected individuals therefore receive one affected gene from each parent who, in turn, is heterozygous for the condition, having one normal and one abnormal gene; heterozygotes, although possessing only half the normal activity of the affected enzyme, rarely display clinical manifestations and can only be identified by meticulous analytical procedures.

The sequence of amino acids in each polypeptide chain occurring in an enzyme protein is coded by the sequence of purine and pyrimidine bases in the deoxyribonucleic acid (DNA) of the corresponding gene. Mutation involves a change in the base sequence of DNA, and Harris [1229] has classified the change in amino acid sequence of the enzyme protein thus brought about as leading to the following functional and structural derangements:

(1) Synthesis of a protein with defective catalytic properties resulting in kinetic modifications, typified by elevated $K_M$ values, and therefore apparent 'loss' of activity under physiological conditions. An example of this type of process is shown in *Figure 1.13*, where Lineweaver–Burk plots for argininosuccinate synthetase from a patient with citrullinaemia reveal the $K_M$ for citrulline to be two orders of magnitude higher than that of normal subjects [1230].

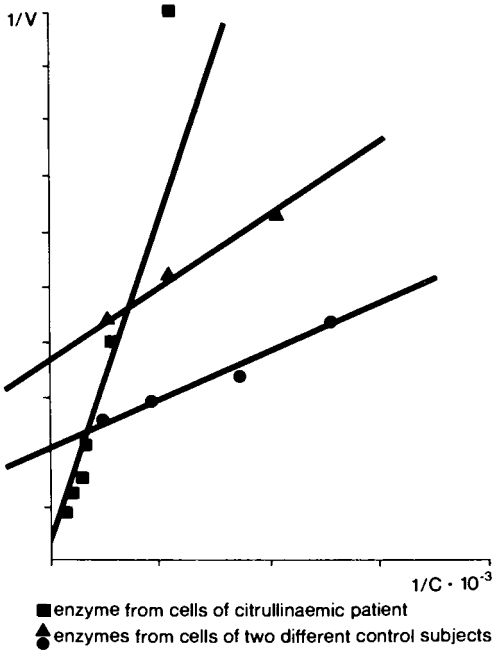


*Figure 1.13. Lineweaver–Burk plots derived from measurements of reaction velocities* (V) *of argininosuccinate synthetase at various concentrations* (C) *of citrulline. The enzyme was studied in extracts of fibroblast cells grown in tissue culture and derived from a patient with citrullinaemia and from appropriate controls. Estimated* $K_m$ *for control enzyme was* $4 \times 10^{-4}$ *M and for citrullinaemic enzyme was approximately* $5 \times 10^{-2}$ *M [1230]*

(2) Synthesis of an enzyme protein with unaltered catalytic properties but decreased stability. Erythrocyte glucose 6-phosphate dehydrogenase deficiency provides examples of this phenomenon; in contrast to the half-life of 62 days attributed to the normal red cell enzyme, the half-lives for the commonest negro variant and Mediterranean variant are 12 days and one day respectively [1231, 1232].

(3) Complete failure of enzyme synthesis. This is probably the commonest of the three mechanisms.

## SPECIFIC ABNORMALITIES

The range of diseases and disorders falling within the scope of this Section is so wide as to cover all tissues, organs, and systems. The following brief summary should assist in orientating the reader.

*Congenital erythrocyte enzymopathies*

These conditions generally manifest as non-spherocytic haemolytic anaemias, or haemolytic anaemia developing after exposure to certain drugs. The main deficiencies in this category are listed in *Table 1.11.* Diagnosis depends upon measuring the enzyme activity of the erythrocytes of suspected subjects, as free from contamination by white cells and platelets as possible, and preferably during a quiescent phase of the disease. Glucose 6-phosphate dehydrogenase deficiency is relatively common, and is inherited as an X-chromosome linked disease. All the other conditions listed in *Table 1.11* are rare and, so far as is known, are inherited as autosomal recessive diseases. A series of fluorescence spot tests has been developed for several of these conditions [1245]. They seem very suitable as screening procedures. The disease does not generally give rise to abnormalities in other tissues, and the main therapeutic problem lies in tiding the patient through a crisis by means of blood transfusion, and avoiding precipitating factors.

Table 1.11. SOME CONGENITAL ERYTHROCYTE ENZYMOPATHIES

| *Enzyme deficiency* | *EC number* | *References* |
|---|---|---|
| Glucose 6-phosphate dehydrogenase | 1.1.1.49 | 1231–1233 |
| 6-phosphogluconate dehydrogenase | 1.1.1.44 | 1234, 1235 |
| Glutathione reductase | 1.6.4.2 | 1236 |
| Pyruvate kinase | 2.7.1.40 | 1237–1239 |
| Triosephosphate isomerase | 5.3.1.1. | 1240 |
| Phosphofructokinase | 2.7.1.11 | 1241, 1242 |
| Hexokinase | 2.7.1.1 | 1243, 1244 |

*Lysosomal enzyme deficiencies*

This group of diseases arises as a consequence of a special type of inborn error specifically affecting intralysosomal digestion. Generally, only one lysosomal enzyme is deficient in any one disease. Depending upon the natural substrate for that enzyme, its deficiency gives rise to the accumulation of macromolecular materials in affected tissues, especially the liver, spleen, and central nervous system. These diseases have therefore in the past been called 'storage diseases', and classified according to the storage material which accumulates, viz. lipid, glycolipid, polysaccharide, and mucopolysaccharide.

Table 1.12. CLASSIFICATION OF GLYCOGEN STORAGE DISEASES

| *Type* | *Eponym* | *Enzyme deficiency* | *EC number* | *References* |
|---|---|---|---|---|
| I | Von Gierke | Glucose 6-phosphatase | 3.1.3.9 | 1251 |
| II | Pompe | α-1,4-glucosidase | – | 1252 |
| III | Cori | Amylo-1,6-glucosidase | 3.2.1.33 | 1253, 1254 |
| IV | Andersen | α-1,4-glucan: α-1,4-glucan 6-glucosyl transferase | 2.4.1.18 | 1255, 1256 |
| V | McArdle | Muscle glycogen phosphorylase | 2.4.1.1 | 1257, 1258 |
| VI | Hers | Liver glycogen phosphorylase | 2.4.1.1 | 1259, 1260 |
| VII | – | Muscle phosphofructokinase | 2.7.1.11 | 1261 |
| VIII | – | Liver phosphorylase *b* kinase | 2.7.1.38 | 1262, 1263 |

Although the term 'lipomucopolysaccharidoses' has been coined to emphasize the unity underlying their aetiology [1246], it is appropriate to consider them on a more selective basis. One group, the glycogen storage diseases, is due to failure of glycogen break-down [1247]. A large number of enzymes are involved in this process, and a list of the diseases occasioned by their absence or deficiency is presented in *Table 1.12.* The first two conditions are severe and eventually fatal; the others are more benign. Not all of the enzymes listed in this table are truly lysosomal but it is convenient to group these diseases together. There is no known therapy for these diseases.

Those diseases giving rise to lipid or glycolipid accumulation are termed 'lipidoses' or 'gangliosidoses', whereas those associated with mucopolysaccharide accumulation are termed 'gargoylism'. The main chemical features of these conditions and the deficient enzymes are listed in *Figure 1.14* which is taken from the review by Brady [1248]. All are severe and ultimately fatal. Little in the way of treatment can currently be offered, although some experimental approaches hold out a degree of promise [1249, 1250]. Pre-natal diagnosis followed by therapeutic abortion is becoming increasingly important in limiting the number of subjects born with these diseases.

*Disorders of amino acid metabolism*

These are among the commoner of the congenital enzyme deficiency states. They lead to accumulation of amino acids, or, in some instances, products of amino acid catabolism (as in phenylketonuria) in the tissues

**CONSTELLATION OF METABOLIC DISEASES IS CHARACTERIZED BY INABILITIES TO DEGRADE SPHINGOLIPIDS**

| Disease | Major sphingolipid accumulated | Enzyme defect |
|---|---|---|
| Niemann Pick | Cer – PChol<br>Sphingomyelin | Sphingomyelinase |
| Gaucher | Cer – β – Glc<br>Ceramide glucoside (glucocerebroside) | β-Glucosidase |
| Krabbe | Cer – β – Gal<br>Ceramide galactoside (galactocerebroside) | β-Galactosidase |
| Metachromatic Leukodystophy | Cer – β – Gal<br>$^{3}OSO_3$<br>Ceramide galactose 3 sulfate (sulfatide) | Sulfatidase |
| Fabry | Cer – β – Glc – β – Gal – β – Gal<br>Ceramide trihexoside | β-Galactosidase |
| Tay-Sachs | Cer – β – Glc – β – Gal – β – NAc Gal<br>NAcNA<br>Ganglioside $GM_2$ | Hexosaminidase |
| Generalized Gangliosidosis | Cer – β – Glc – β – Gal – β – NAc Gal – β – Gal<br>NAcNA<br>Ganglioside $GM_1$ | β-Galactosidase |

Cer = N-Acyl Sphingosine (Ceramide) NAcNA = N-Acetylneuraminic Acid

*Figure 1.14. Correlation of sphingolipid accumulation and enzymatic defect in certain storage diseases associated with lysosomal enzyme deficiencies [1248]*

and body fluids, with overflow into the urine. The more important of these conditions are listed in *Table 1.13.* The definitive diagnosis is made by demonstrating absence of the enzyme concerned; a variety of cells and tissues may be used for this purpose. Some of these conditions are capable of pre-natal diagnosis with a view to termination of pregnancy or early institution of therapy. This consists of strict dietary control aimed at preventing ingestion of the amino acid(s) whose metabolism is blocked. It is not altogether certain how effective such treatment is, and many series report disappointing experiences in the long term follow-up of affected cases.

## *Disorders of carbohydrate metabolism*

A list of the more important conditions in this field dependent upon

Table 1.13. SOME HEREDITARY DISORDERS OF AMINO ACID METABOLISM

| *Disease* | *Enzyme deficiency* | *EC number* | *Deficient tissues* | *References* |
|---|---|---|---|---|
| Phenylketonuria | Phenylalanine hydroxylase | 1.14.3.1 | Liver | 1264, 1265 |
| Tyrosinaemia | *p*-OH-phenyl pyruvic acid oxidase | 1.14.2.2 | Liver | 1266, 1267 |
| Alcaptonuria | Homogentisic acid oxidase | 1.13.1.5 | Liver and kidney | 1268 |
| Histidinaemia | Histidine $\alpha$-deaminase | – | Liver and skin | 1269, 1270 |
| Argininosuccinic aciduria | Argininosuccinic acid lyase | 4.3.2.1 | Liver, kidney, erythrocytes | 1271, 1272 |
| Homocystinuria | Cystathionine synthase | – | Liver | 1273–1275 |
| Maple syrup urine disease | Branched-chain keto acid decarboxylase | – | Leucocytes | 1276 |
| Albinism | *o*-Diphenol oxidase | 1.10.3.1 | Skin | 1277 |

congenital deficiency of an enzyme is compiled in *Table 1.14.* Considerable interest was aroused by the intestinal disaccharidase deficiencies which appeared to explain the presence of intractable diarrhoea and malabsorption in a number of cases in whom no other factor could be

Table 1.14. SOME HEREDITARY DISORDERS OF CARBOHYDRATE METABOLISM

| *Disease* | *Enzyme deficiency* | *EC number* | *Deficient tissues* | *References* |
|---|---|---|---|---|
| Lactose intolerance | Lactase | 3.2.1.23 | Small intestine | 1278–1280 |
| Sucrose intolerance | Sucrase: isomaltase | 3.2.1.26 | Small intestine | 1281, 1282 |
| Galactosaemia | Galactose 1-phosphate uridyl transferase | 2.7.7.10 | Liver, erythrocytes, small intestine | 1283 |
| Fructose intolerance | Fructose 1-phosphate aldolase | 4.1.2.7 | Liver, kidney, small intestine | 1284 |
| Fructosuria | Fructokinase | 2.7.1.4 | Liver | 1285 |
| Pentosuria | Xylitol: NADP oxidoreductase | 1.1.1.10 | Erythrocytes | 1286 |

incriminated. It is now known that disaccharidase deficiencies can arise as a feature of several diseases causing damage to small intestinal mucosa [1287–1289], but these secondary deficiencies are usually multiple, whereas the true primary congenital deficiencies are restricted to a single enzyme.

Galactosaemia is a condition which holds great interest as it was among the first diseases to be explained by a congenital enzyme deficiency [1290]. This enzyme, galactose 1-phosphate uridyl transferase, is deficient in all those tissues in affected white homozygous subjects in which it has been measured, including erythrocytes. Because the latter are easily obtainable, measurement of erythrocyte galactose 1-phosphate uridyl transferase activity is the method of choice in establishing the diagnosis and in family studies aimed at heterozygote detection [1291, 1292]. Negroes who are homozygous for galactosaemia appear to show residual transferase activity in liver and intestinal mucosa, have a limited capacity to metabolize galactose, and do not develop such florid clinical features as affected white children [1293, 1294]. Another mutation, termed the Duarte Variant, results in diminished transferase activity in the erythrocytes to half-normal in the homozygote and three-quarters-normal in the heterozygote [1295]; however no clinical abnormalities arise in this condition. The variant enzyme can be distinguished from the normal enzyme only by virtue of its faster mobility on starch-gel electrophoresis [1296, 1297].

Screening programmes for galactosaemia based on qualitative tests for erythrocyte galactose 1-phosphate uridyl transferase have been developed [1298–1302], but the efficacy of such programmes is in doubt because the incidence of the disease seems to be lower than was originally thought. Figures vary from 1 in 35000 to 1 in 70000 live births and the reported salvage rates have been very low [1302–1305].

An even rarer disease, associated with the presence of excess galactose in the blood, is due to deficiency of the enzyme galactokinase [1306]. This manifests itself in cataracts, but the severe organ damage – to liver, brain, and kidney – occurring in untreated classical galactosaemia does not occur in galactokinase-deficient subjects.

*Miscellaneous congenital enzymopathies*

A list of the more important diseases attributable to congenital enzyme deficiences not classifiable in the previous 4 categories is presented in *Table 1.15.* Two of these are not strictly enzyme deficiencies but they do have strong enzymological implications and the diagnosis may rest with

Table 1.15. OTHER HEREDITARY ENZYME DEFICIENCIES

| *Disease* | *Deficient enzyme/protein* | *Main clinical features* | *Deficient tissues* |
|---|---|---|---|
| Antitrypsin deficiency | α-1-antitrypsin | Pulmonary emphysema, neonatal hepatitis, juvenile cirrhosis | Serum |
| Crigler–Najjar disease | UDP-glucuronyl transferase | Severe jaundice | Liver |
| Gilbert's disease | UDP-glucuronyl transferase | Mild jaundice | Liver |
| Lesch–Nyhan syndrome | Hypoxanthine-guanine phosphoribosyl transferase | Hyperuricaemia, self-mutilation and mental deficiency | Leucocytes, erythrocytes, skin, liver |
| LCAT deficiency | Lecithin-cholesterol acyl transferase | Corneal opacity, anaemia and lipid disorders | Serum |
| Hypophosphatasia | Alkaline phosphatase | Bone disorder and urinary phosphoethanol-amine excretion | Serum, liver, bone |
| Acute intermittent porphyria | Uroporphyrinogen I synthetase | Severe neurologic and gastro-intestinal manifestations | Liver |
| Refsum's disease | Phytanic acid oxidase | Retinitis pigmentosa, cerebellar ataxia, peripheral neuropathy | Skin |
| Suxamethonium sensitivity | Cholinesterase | Scoline anpoea | Serum |
| Wilson's disease | Caeruloplasmin/copper oxidase | Hepato-lenticular degeneration | Serum |
| Adrenogenital syndromes | Steroid 21-hydroxylase | Virilization and salt-losing state | Adrenals |
| | Steroid 11β-hydroxylase | Virilization and hypertension | Adrenals and gonads |
| | Steroid 17α-hydroxylase | Hypogonadism and hypertension | Adrenals and gonads |

the routine laboratory. In anti-trypsin deficiency, a major component of the serum α-1 globulin fraction is absent. This has the property of inhibiting trypsin and other proteases of the peripheral blood, and its absence is held responsible for the pulmonary emphysema that develops in these patients as young adults [1307, 1308]. In one form of the

condition, the protein, while absent from the serum, is synthesised in large amounts and stored in the liver, presumably because a transport defect prevents its passage out of the liver cell. Infantile cirrhosis, often but not invariably preceeded by a form of neonatal hepatitis, is the hallmark of this condition [1309, 1310]. Nine variant alleles and 17 phenotypes of $\alpha$-1-anti-trypsin have been identified, but only one genotype, designated PiZZ, is associated with homozygous deficiency [1311].

In Wilson's disease, the defect has not been established with certainty. Increased deposition of copper takes place in those organs–the liver, lens of the eye, and central nervous system–whose damage gives rise to the clinical features of the syndrome, and there is excess of free copper in the blood serum (but reduction in total serum copper concentration) and increased urinary copper excretion [1312, 1313]. Most patients have reduced levels of caeruloplasmin, the copper-transport protein of the serum, which, until the advent of recent immunochemical techniques was traditionally measured as serum copper oxidase activity [1314, 1315]. However, some subjects with this disease have normal caeruloplasmin levels. No difference between the caeruloplasmin of normal subjects and patients with Wilson's disease can be detected on peptide mapping [1316], but it has been claimed that differences in binding constants exist between caeruloplasmin from the two groups.

The status of acute intermittent porphyria as an inherited enzyme deficiency state is somewhat tentative. It is widely agreed that the liver in affected subjects contains a great increase in $\delta$-amino laevulinic acid synthetase, leading to overproduction of this compound and its appearance in excessive concentration in the urine [1317, 1318]. More recently, hepatic levels of uroporphyrinogen I synthetase were found to be reduced to less than 50% of the levels in normal subjects and patients with other forms of porphyria [1319]. It remains to be established that this reduction is occasioned by a primary genetic defect and is capable of explaining the multifarious chemical features of this disease.

The congenital unconjugated hyperbilirubinaemias represent another group of diseases where deficiency of an enzyme – in this instance, UDP-glucuronyl transferase – is thought to represent the primary genetic lesion, although final proof is still lacking. The evidence for this view seems more convincing in the severest disease of this group, the Criggler–Najjar syndrome [1320]. Arias has segregated a sub-type of the above which runs a less severe course and in which only a partial deficiency of the enzyme can be demonstrated [1320]. The mildest of the group,

Gilbert's disease, is usually asymptomatic, and as it occurs in about 1% of the general population, it ranks as one of the commonest of all congenital disorders. Its cause has been the subject of great debate: increased haemolysis, defective uptake of bilirubin by the liver cell, or blockage of hepatic uptake of bilirubin by a hypothetical inhibitor, have all been postulated. The most convincing hypothesis, based upon detailed analysis of a large series of cases, points to a modest deficiency of UDP-glucuronyl transferase which can only be recognised when patients with Gilbert's disease are compared with patients having comparable degrees of icterus from other causes [1321]. This is necessary because a high bilirubin concentration induces increased synthesis of the transferase, and this induction masks the underlying deficiency in the icteric patient.

The Lesch–Nyhan syndrome is a severe condition characterised by mental deficiency, bizarre and characteristic self-mutilation, and disordered purine metabolism. The basic defect is an absence of the enzyme hypoxanthine-guanine phosphoribosyl transferase, the deficiency having been demonstrated in a wide variety of cells and tissues [1322–1324]. Genetic heterogeneity within the Lesch–Nyhan syndrome has been claimed as a consequence of finding in an affected subject an atypical transferase with a very high $K_M$ value which yielded up to 34% of the activity of the normal enzyme at high substrate concentration [1325].

Deficiency of serum cholinesterase occurs as an acquired condition in liver disease [1087, 1326] and also in the course of therapy with certain drugs [1327], while it has been claimed that schizophrenia is associated with raised levels of this enzyme [1328]. Its diagnostic utility in these conditions is not very impressive. Greater interest attaches to the rarer congenital deficiencies of serum cholinesterase, in which the patient enjoys an entirely asymptomatic existence except when exposed to muscle relaxants of the succinyl choline (scoline) type, as occurs during general anaesthesia. Difficulty in hydrolysing this compound, which is rapidly broken down by the serum cholinesterase of normal subjects, leads to prolonged and potentially fatal apnoea after anaesthesia, although maintenance of artificial ventilation is all that is necessary in the way of treatment, since the drug is ultimately metabolised and spontaneous respiration regained.

The deficiency of cholinesterase activity in the serum is in most instances relative rather than absolute and arises not through absence of the enzyme but because of the presence of an abnormal genetically determined molecular variant. There are two gene loci for cholinesterase. One of these, $E_2$, is associated with an additional cholinesterase band on

electrophoresis but with no other abnormality [1329]. The other locus, $E_1$, is associated with the clinically significant molecular variants. The usual enzyme is designated $E_1^u$. The commonest abnormal variant, the atypical enzyme, is designated $E_1^a$; it has low activity against scoline, but is highly resistant to the compound dibucaine which is a powerful inhibitor of the usual enzyme. A number of other inhibitors, including the synthetic compound R02-0683, the dimethyl carbamate of (2-hydroxy-5-phenylbenzyl)trimethyl ammonium bromide, have been used to distinguish between the usual and atypical enzyme. The use of sodium chloride and formaldehyde has been proposed as a means of distinguishing the two enzymes [1330], and it has been claimed that differentiation can also be achieved by assaying the activity in Tris and phosphate buffers [1331]. The second variant, the fluoride-resistant enzyme, is designated $E_1^f$. This is slightly sensitive to scoline, being about 10% less active than the usual enzyme but is resistant to inhibition by fluoride ions which inhibit the usual enzyme. Differential inhibition in tris and phosphate buffers has also been advocated as a means of distinguishing the usual and fluoride-resistant enzymes [1332]. All three gene products exist in homozygous and heterozygous forms, dual heterozygosity for both atypical and fluoride-resistant genes being well documented. The behaviour of the various cholinesterase $E_1$ phenotypes with the inhibitors described is represented in *Figure 1.15* which is taken from the review by King [1333]. Another variant, known as the silent enzyme, and designated $E_1^s$, is devoid of activity and therefore its sensitivity to inhibitors cannot be ascertained. The temperature–activity relationships of the molecular variants of cholinesterase show marked differences. The atypical homozygote is maximally active at 32–34°C, the usual homozygote at 50–51°C, and the fluoride-resistant homozygote and the various heterozygotes at intermediate temperatures [1333–1335]. Further information on these fascinating genetic aberrations may be obtained in appropriate reviews [1336, 1337].

## PRE-NATAL DIAGNOSIS

With few exceptions, little can be done to cure congenital enzyme deficiency diseases. Some are amenable to treatment which often has to continue for life. Blood transfusions and avoidance of precipitating factors such as drugs or certain foodstuffs will help patients with erythrocyte enzymopathies leading to haemolytic anaemia. Disaccharidase deficiencies, galactosaemia, and some of the disturbances of amino acid

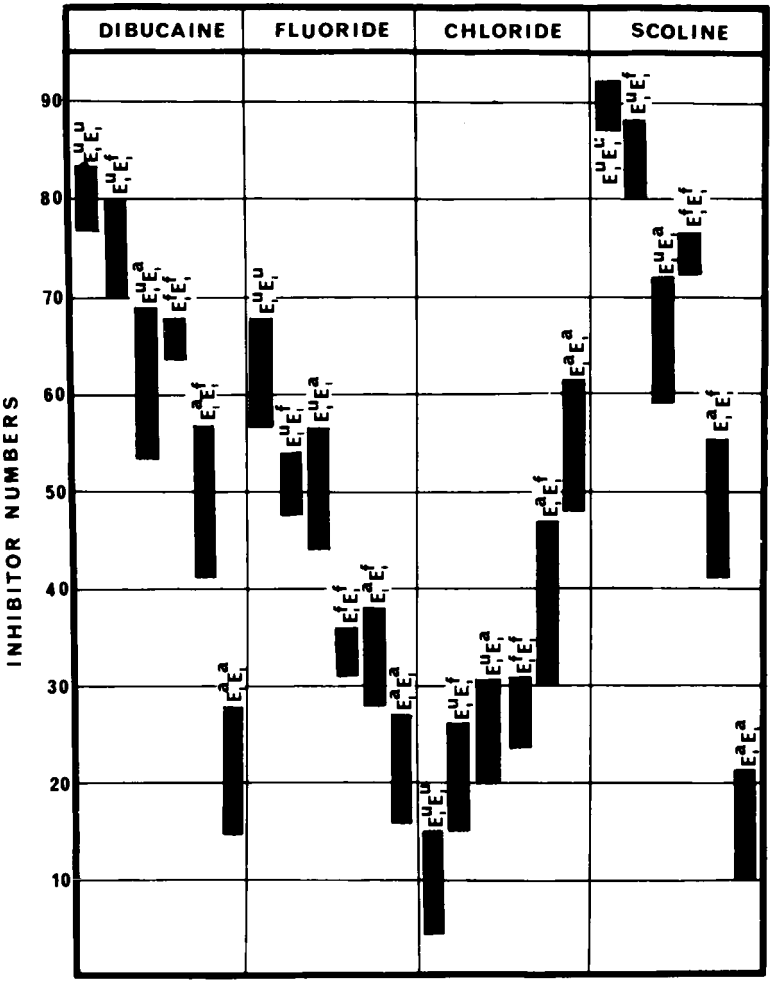


*Figure 1.15. Range of inhibitions exhibited by the different phentotypes of serum cholinesterase at the $E_1$ locus with various inhibitors [1333]. Nomenclature explained in text*

metabolism can be controlled by removing from the diet the constituent whose altered metabolism gives rise to the toxic intermediates. For most, not even this type of therapy is possible, and many of these diseases give rise to crippling deformities, mental deficiency, and early death. Changes in social mores and medical ethics have made it possible to offer therapeutic abortion to mothers bearing affected foetuses, and advances

in enzyme technology and tissue-culture procedures have enabled such foetuses to be recognised *in utero* before 24 weeks of gestation and at a time when the risks of therapeutic abortion are acceptable. More than 50 such conditions can now be diagnosed at this time.

The technique has been pioneered by Nadler [1338–1342], and the subject has been reviewed by Davidson and Rattazi [1343] and, more extensively, by Milunsky [1344]. The essential steps are outlined in *Figure*

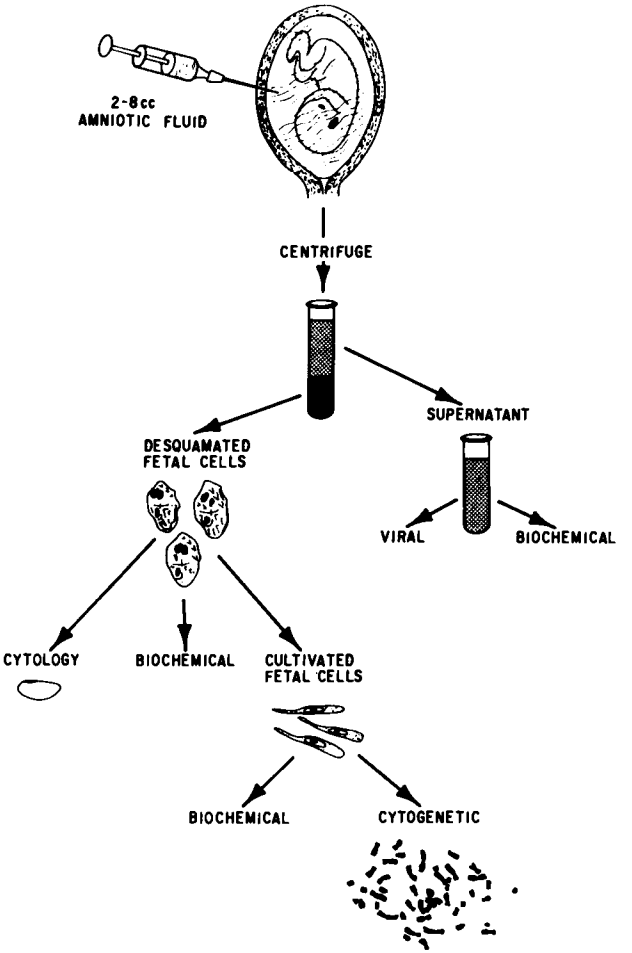


*Figure 1.16. General scheme for utilization of amniotic fluid for prenatal detection of genetic disorders [1340]*

*1.16.* Amniotic fluid is obtained by amniocentesis and centrifuged. Biochemical tests may be carried out on the supernatant fluid and on the foetal cells, but more usually diagnosis depends upon culturing the latter to improve the yield and demonstrating absence of the enzyme directly, or indirectly by showing inability of the cells to metabolise a compound above the enzymatic block. Among the conditions so diagnosed, mention may be made of Fabry's disease [1345], Krabbe's leukodystrophy [1346], Lesch–Nyhan syndrome [1347, 1348], Tay–Sachs disease [1349], Niemann–Pick disease [1350], and Gaucher's disease [1351]. Screening of pregnancies is only feasible where, as a consequence of relationship with a known propositus, the risk can be quantified. Generally, the pregnant mother will have given birth to an affected child by an earlier pregnancy. The biochemical techniques are expensive and demanding, being well beyond the scope of routine hospital laboratories. The policy of the Department of Health in the United Kingdom is to establish national reference centres where these tests can be performed.

## CONCLUSION

The foregoing text has covered the most important aspects of clinical enzymology from the standpoint of diagnostic enzyme assays and their impact upon patient care. One aspect of enzymes in the routine laboratory that has not yet been mentioned is their role in substrate determinations. Enzymatic assay techniques are being increasingly applied to the determination of commonly-required constituents of serum; these techniques make use of the high specificity of enzymes for their substrates and the sensitivity of many of the spectral changes brought about by specific enzyme action, in particular, those involving changes in absorbance or fluorescence of oxidised and reduced pyridine nucleotides linked to dehydrogenases.

Those constituents of clinical interest which are measurable in this way include alcohol [1352], pyruvate and lactate [1353], acetoacetate and $\beta$-hydroxybutyrate [1354], amino acids [1355], ammonia [1356, 1357] and galactose [1358]. Glucose may be measured using glucose oxidase and peroxidase linked to chromogenic oxygen acceptors [1359–1361] or by a coupled reaction involving linkage of hexokinase to glucose 6-phosphate dehydrogenase [1362, 1363]. The use of urease in the determination of urea was one of the earliest analytical applications of enzymes as reagents, but more recently, a coupled reaction with glutamate dehyd-

rogenase has been gaining in popularity [1364, 1365]. Uric acid may be measured using the enzyme uricase [1366], and fully enzymatic methods for determining triglycerides [1367] and cholesterol [1368] are now available. D-Glucaric acid is usually determined by the inhibitory effect of its lactone on $\beta$-glucuronidase [1369], and the use of immobilised enzymes for assay purposes has led to the evolution of a series of 'enzyme electrodes' [1370].

These methods are in no sense esoteric. In many instances, they are the methods of choice for that particular estimation, providing an accuracy, a precision, an ease of operation, and a suitability for automation and mechanisation far beyond the scope of traditional chemical methods, which they are replacing on an increasing scale. The clinical chemist is being called upon to master technical aspects of enzymology not required by his predecessors, while the clinician has at his disposal a larger range of diagnostic tests than ever before. It matters little to him how the blood urea is estimated as long as he has confidence in the results. Interpretation of data for enzyme activities faces him with greater problems. Individual values often have little meaning or they can point to too many conflicting and irreconcilable possibilities. Serial determinations of the same enzyme may be more informative, but the information may be restricted. Profiles of different enzymes reveal disease-specific patterns that are an unquestionable aid to diagnosis. The task for the future will lie as much in the recognition of these patterns, choosing the appropriate profile for the clinical problem at hand, discarding useless enzyme tests and making the best use of the tests now available, as in the development of new enzyme procedures.

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# 2 The Release of Pharmacologically Active Substances in Parasitic Infections

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## INTRODUCTION

In recent years, considerable attention has been devoted to pathological processes in parasitic diseases with a view to understanding the mechanism of development of the signs and symptoms which they cause. This type of study has involved parasitologists, medical and veterinary personnel, immunologists, biochemists, pharmacologists and pathologists. This review is concerned with the release of pharmacologically active substances. Since a very wide field is involved, it is important to define exactly what is to be covered. The substances reviewed include the kinin system, histamine, 5-hydroxytryptamine, slow reacting substance of anaphylaxis, catecholamines, acetylcholine, prostaglandins, angiotensin and substance P. No studies on angiotensin or substance P have been undertaken so far. Under parasitic infections, we include protozoal and helminth infections of man and other animals but exclude bacterial and viral infections. A large field is being covered, but in many cases the amount of information is small and therefore it will be difficult to come to conclusions on the role of these agents in disease processes. To date, no reviews on this topic have been produced and the available information is widely scattered throughout many scientific journals. An attempt has therefore been made to collate this information and draw what conclusions are possible.

## PARASITIC INFECTIONS

In order to understand the significance of studies on the release of pharmacologically active substances, some basic parasitology must be considered. The number of parasitic infections studied so far is small and is by no means representative of all orders and classes of parasites. Those parasites which have been investigated tend to be either infections of economic importance to man or his domestic animals or convenient laboratory models. *Tables 2.1* and *2.2* summarise the basic parasitological data of the main organisms in the review.

### BABESIA

The genus *Babesia* are protozoan parasites that infect erythrocytes of vertebrates and are transmitted by ixodid ticks. 71 Species are found in this genus [1], some of which are important agents of disease of domestic animals. *B. argentina* causes disease in cattle characterised by fever, anorexia, diarrhoea, vomiting, anaemia, haemoglobinuria, uraemia, jaundice and brain involvement. Death usually results in 1–2 weeks unless treated. *B. rodhaini* is a convenient laboratory model.

### MALARIA

Malaria parasites are protozoa belonging to the genus *Plasmodium*. Over 100 different parasites have been described [4]. Mammalian parasites are transmitted almost entirely by *Anopheles* mosquitoes while most avian malarias are transmitted by Culicine mosquitoes. Four species are considered as the cause of major disease of man: *Plasmodium falciparum*, *P. malariae*, *P. ovale* and *P. vivax*. Until recently, none of these parasites could be studied under laboratory conditions and so monkey and rodent parasites have been used as models. It has recently been shown, however, that several human parasites also infect the owl monkey (*Aotus trivirgatus*) [9–11]. The life cycle is complex and exhibits two types of schizogony in the vertebrate host, one in the erythrocytes and another in the tissues. Sporogony also occurs in the mosquito. The disease is characterised by periodic bouts of fever occurring at about 48 h intervals for tertian malaria (*P. falciparum*, *P. ovale* and *P. vivax*) and at 72 h intervals for quartan malaria (*P. malariae*). The periods of fever and

Table 2.1. PROTOZOAN PARASITES

| *Parasite* | *Natural vertebrate host* | *Major experimental host* | *Vector* | *Sites of parasitism* | *Disease in experimental host* | *Distribution* | *References* |
|---|---|---|---|---|---|---|---|
| Family: Babesiidae | | | | | | | |
| *Babesia argentina* | Cattle | Cattle | *Boophilus microplus* | Erythrocyte | Acute | Australia, South America, Madagascar | 1, 2 |
| *Babesia rodhaini* | Thicket rat | Mouse, rat | Unknown | Erythrocyte | Acute | Zaire | 3 |
| Family: Plasmodiidae | | | | | | | |
| *Plasmodium berghei* | Thicket rat | Mouse, rat | *Anopheles dureni* | Erythrocyte | Chronic | Zaire | 4 |
| *Plasmodium knowlesi* | Monkeys | Rhesus monkey | *Anopheles* mosquitoes | Erythrocyte | Acute | South East Asia | 4 |
| Family: Trypanosomatidae | | | | | | | |
| *Trypanosoma (Trypanozoon) brucei* | Domestic and wild animals | Rat, mouse rabbit | Tsetse flies | Blood, connective tissue | Chronic or acute | West, Central and East Africa | 5 |
| *Trypanosoma (Trypanozoon) rhodesiense* | Man, domestic and wild animals | Rat, mouse rabbit | Tsetse flies | Blood, connective tissue | Chronic or acute | East and Central Africa | 5 |
| *Trypanosoma (Duttonella) vivax* | Domestic and wild animals | Sheep, rat | Tsetse and other biting flies | Blood, lymph nodes | Chronic or acute | West, Central and East Africa, Central and South America, West Indies, Mauritius | 5 |
| *Trypanosoma (Herpetosoma) lewisi* | Rat | Rat | Flea | Blood | Nonpathogenic | Cosmopolitan | 5 |

Table 2.2. HELMINTH PARASITES [6–8]

| *Parasite* | *Definitive host* | *Experimental host* | *Intermediate host/vector* | *Larval migration in definitive host* | *Parasitisation by adult* | *'Self-cure' phenomenon* | *Distribution* |
|---|---|---|---|---|---|---|---|
| Class: Nematoda | | | | | | | |
| Order: Strongylida | | | | | | | |
| Family: Trichostrongylidae | | | | | | | |
| *Haemonchus contortus* | Sheep | – | – | Abomasal wall | Abomasum lumen | + | Cosmopolitan |
| *Trichostrongylus colubriformis* | Sheep cattle | Guinea pig | – | Intestinal epithelium | Small intestine lumen | + | Cosmopolitan |
| *Nippostrongylus brasiliensis* | Rat | – | – | Skin, circulatory system, lungs, small intestine | Small intestine lumen | + | |
| Family: Strongylidae | | | | | | | |
| *Strongylus vulgaris* | Horse | – | – | Large intestinal wall, circulatory system, mesenteric artery | Caecum and colon lumen | – | Cosmopolitan |
| Order: Trichurida | Carnivore | Rat | Other carnivores | Blood stream, muscle | Small intestine, mucosa and crypts | – | Cosmopolitan |
| Family: Trichinellidae | | | | | | | |
| *Trichinella spiralis* | | | | | | | |
| Order: Ascarida | | Rat | – | Small intestine wall, liver, blood, stream, lungs and small intestine | Small intestine lumen | – | Cosmopolitan |
| Family: Ascaridae | | | | | | | |
| *Ascaris lumbricoides* | Man | | | | | | |
| *Ascaris suum* | Pig | | | | | | |
| Order: Filarida | | | | | | | |
| Family: Filaridae | Dog | – | Mosquito | Blood stream | Right ventricle, pulmonary artery | – | Tropics and subtropics |
| *Dirofilaria immitis* | | | | | | | |

associated phenomena are associated with schizogony of the parasite [12]. Other symptoms include anaemia, enlargement of the spleen and liver and often brain involvement.

## TRYPANOSOMES

Trypanosomes are flagellated protozoa of the family Trypanosomatidae which parasitise the blood and tissues of many different hosts including plants, insects, fish, birds, reptiles, amphibians and mammals [2, 3, 5, 13]. In Africa they cause sleeping sickness in man (*Trypanosoma rhodesiense* in East and Central Africa, *T. gambiense* in West Africa) and Nagana in cattle (*T. congolense*, *T. vivax* and *T. brucei*). Domestic and wild animals are known to be reservoir hosts for Rhodesian sleeping sickness [14–17]. The parasites undergo a developmental cycle in their vector, the tsetse fly, and are transmitted via the proboscis when the fly takes a blood meal. Gambian trypanosomiasis develops slowly and is characterised by weakness, wasting, lethargy, fever, lymph-glandular involvement and eventually involvement of the central nervous system. Rhodesian sleeping sickness develops more rapidly and death may occur before the central nervous system is seriously involved [12].

## THE INTESTINAL NEMATODES

We shall be considering in detail three Trichostrongylid nematodes (*Haemonchus contortus*, *Trichostrongylus colubriformis* and *Nippostrongylus brasiliensis*). Only the first two of these are parasites of economic importance. The life cycle of *H. contortus* is direct and no intermediate hosts are involved. The eggs are passed in the faeces, hatch on the pasture and develop through three non-parasitic larval stages before becoming the infective larva which is taken up with the grass while the animals are grazing. On reaching the abomasum, further development occurs until the adult stage is reached. A similar life cycle is found with *T. colubriformis* although it parasitises the small intestine. *N. brasiliensis*-infective larvae actively penetrate the skin and migrate to the small intestine where they develop into the adult form. The latter two parasites are convenient laboratory models.

*Strongylus vulgaris* is a member of the family Strongylidae found primarily in the large intestine of the horse. After ingestion the fourth stage larvae undergo extensive abdominal migration before parasitising the large intestine of the host as adult worms.

One of the commonest of all nematodes is the round worm *Ascaris lumbricoides* which is a cosmopolitan parasite of man, while closely related species infect a variety of other animals. The adults inhabit the intestine and the eggs pass out with the faeces. Infection of a second host occurs by eating or drinking polluted food or water containing the embryonated eggs. After hatching in the intestine the worms undergo an extensive tissue migration before returning to become established in the intestine. Heavy infestations may result in symptoms and signs due to the mechanical effects of the presence of worms and are typified by enteritis and parasitic pneumonia.

### OTHER NEMATODES

Two other nematodes will be considered. *Trichinella spiralis* family Trichinellidae has a widespread distribution and an extensive range of hosts including man, pig, rat, polar bears, cats, dogs, seals, walruses and whales [6–8]. Infection occurs by eating uncooked meat containing the encapsulated larvae. The worms are released in the duodenum and migrate into the intestinal villi. The female worms are viviparus and lay first stage larvae that pass into the host's tissues particularly in the voluntary muscle where they develop into encysted forms. The worms can stay in this state for several years but, on being eaten by a second host, maturation into adult stages occurs rapidly.

The final nematode to be considered is *Dirofilaria immitis* superfamily Filaroiodea. It is found in the right ventricle and pulmonary arteries of dogs, cats, wolves, foxes and other carnivores. *D. immitis* is transmitted by mosquitoes which feed on infected animals and pick up the larval form–the microfilaria from the blood. The microfilariae show periodicity in the blood, maximum numbers being found at 18.00 h and minimum numbers at 06.00 h.

## THE KININ SYSTEM

### PHARMACOLOGY OF THE KININ SYSTEM

Since the early observations by Frey and his colleagues in Germany [18, 19] that human urine contained a substance which caused a prolonged

fall in arterial blood pressure when injected intravenously into dogs, a very large literature has developed including many symposia and reviews [20–29].

Kinins are small polypeptides which are formed when specific kinin releasing enzymes (kallikreins) act on protein substrates (kininogens). Three naturally occurring kinins have been isolated from plasma (*Figure 2.1*) but others are found in colostrum [30], urine [31], wasp and hornet venoms [32–34], skin of amphibia [35, 36] and the plasma of reptiles and birds [37–39].

The pharmacological properties of bradykinin are summarized in *Table 2.3* which is taken from Zeitlin [40].

The properties of the other naturally-occurring kinins are similar. However kallidin is only half as effective as bradykinin on isolated guinea pig ileum, but 2–3 times more effective at lowering rat arterial blood pressure [27].

Specific kallikreins (kininogenases) occur in mammalian urine, pancreas, plasma, submaxillary gland, saliva, tears, sweat, lymph and cerebrospinal fluid [27, 40, 41]. The plasma kininogenases are normally present as inactive precursors prekininogenases (prekallikreins) which are

Table 2.3. PHARMACOLOGICAL ACTIONS OF BRADYKININ[a]

*Smooth muscle*

Contracts many isolated smooth muscle preparations including guinea-pig ileum (concentration 1 ng/ml), oestrus rat uterus (concentration 0.1 ng/ml) and human uterine strip 1–10 μg/ml), and relaxes others such as rat duodenum (8 ng/ml).

Generally relaxes human intestinal circular muscle, and inhibits peristalsis.

Produces bronchoconstriction on intravenous injection in the guinea-pig.

*Cardiovascular system*

Intravenous injection (0.05–0.5 μg/kg) produces a fall in arterial pressure.

Causes vasodilation in most vascular beds and a fall in total peripheral resistance.

Intravenous injection of minute amounts causes cutaneous flushing in man.

*Nervous system*

Stimulates sympathetic ganglia and causes release of catecholamines from the adrenal medulla.

Stimulates the pain fibres of an exposed blister base in man (0.1–1.0 μg/ml).

*Local action*

Increases capillary permeability when injected intracutaneously (1–10 ng/ml).

[a]Reproduced by courtesy of Blackwell Scientific Publications Ltd.

| | |
|---|---|
| Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg | Kinin I Bradykinin |
| Lys–Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg | Kinin II Kallidin |
| Met–Lys–Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg | Kinin III |

*Figure 2.1. Amino acid sequence of three naturally occurring plasma kinins*

readily activated by a number of procedures including dilution, contact with glass or acidification [40]. It is outside the scope of this review to detail the knowledge on the kallikreins but it is important to realise that each kallikrein is highly substrate-specific, for example, plasma kallikrein releases bradykinin from kininogen while trypsin and glandular kallikrein release kallidin from kininogen. The situation is complicated even further since it is known that at least two kininogens exist [42–44] and that there are naturally-occurring inhibitors of kallikreins [45].

In addition to the pharmacological effect resulting from the release of kinins, kallikreins have been shown to have direct actions:

(1) Isolated small intestine of dog and cat contracts to pancreatic and urinary kallikreins in vitro [46].
(2) Vasodilation of coronary vessels of rabbit heart [47].
(3) Vasodilation and increased capillary permeability in vivo [20, 23].
(4) Lowering of blood pressure [20].

Kinins have a very short half-life in the blood, less than one complete circulation of the blood [48] because of the presence of kininases. Kallikrein however has a longer half-life. Aminopeptidases break down kallidin to bradykinin [49] while further breakdown is brought about by carboxypeptidases [50]. Chymotrypsin is also an effective kininase but trypsin will not inactivate bradykinin, although it does however release the N-terminal lysine from kallidin and the Met–Lys dipeptide from Kinin III [27].

The inter-relationship of the components of the kinin system are shown in *Figure 2.2.*

## THE KININ SYSTEM IN PROTOZOAL INFECTIONS

Studies on the release of kinins in protozoal infections and their possible importance have largely been carried out by three groups of workers using different parasites. Goodwin, Richards and Boreham have investigated African trypanosome infections using mainly rat, mouse and rabbit models although some comparative studies on cattle and man have been

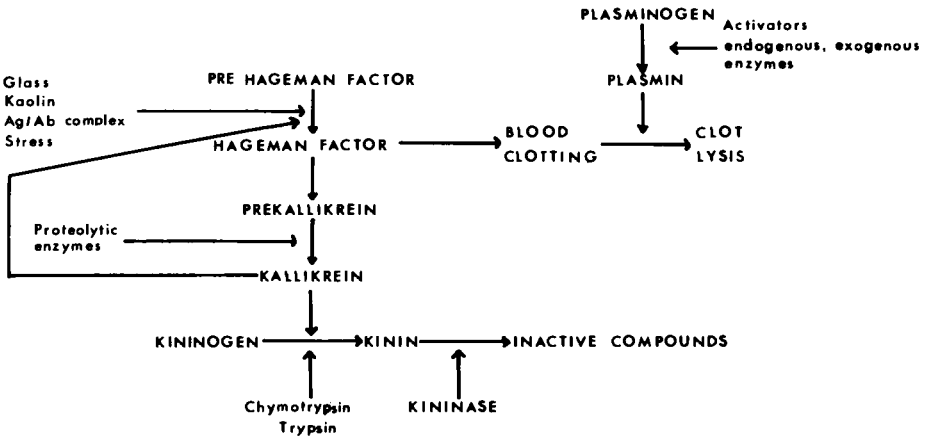


*Figure 2.2. Diagram to show the major component of the kinin system and their interrelationships*

undertaken. Maegraith, Tella and Onabanjo have looked at malaria with particular attention to *P. knowlesi* in monkeys and Mahoney and Wright have studied *B. argentina* infections of cattle. The first two groups have concentrated on kinin and kininogen using bioassay techniques while the third group have investigated the role of kallikrein using biochemical assay methods.

### *Trypanosomes*

During attempts to investigate pathogenic mechanisms in animals inoculated with infective agents Goodwin and Richards [51] found that substances were present in the blood and urine of mice and rats infected with *Babesia rodhaini, Plasmodium berghei, Trypanosoma rhodesiense, Streptococcus pyogenes* and Rift Valley fever virus which caused the contraction of isolated guinea pig ileum and relaxed the rat duodenum. All the infections used were acute, killing the host within 4–8 days. The amount of this substance progressively increased as the infections continued. These authors showed that the active substances were stable to boiling with hydrochloric acid but not alkali, were rapidly destroyed by papain and less rapidly by chymotrypsin but were stable to trypsin and pepsin. It was also shown that the activity on smooth muscle was not antagonised by atropine, mepyramine or lysergic acid diethylamide and it was also unaffected by eserine, iproniazid and bretylium. It was con-

cluded that the substances released were peptides and they suggested that biologically active peptides might be liberated from proteins in all serious illnesses and that their pharmacological activity may cause some of the signs and symptoms of infectious diseases.

This work was followed by studies on human patients with tissue damage resulting from extensive burns [52] and again active peptides and histamine were found in the urine. Richards [53] found that in mice infected with *T. brucei* the kinin activity of plasma increased from $15 \pm 6.9$ ng of bradykinin/ml to $84 \pm 19.1$ ng per ml on the fifth day of the infection just prior to death. Approximately three-fold increases were noted in the kinin content of the ears, skin and feet of these infected mice. The active substance was shown to behave in a similar fashion to the substance described in the earlier work [51] but in addition it was noted that extracts caused a prolonged fall in blood pressure in the anaesthetised rat. The kininogen content of the mouse plasma progressively decreased during the infection. No real attempt was made to explain the mechanism of kinin release and since the acute infection of mice bears little relationship to the chronic disease in man or cattle subsequent studies were undertaken on chronic infections.

In rabbits infected with *T. brucei*, a chronic disease develops which is characterised by successive waves of parasites of different antigenic types [54]. Death usually occurs after 4–8 weeks depending upon the strain used. Plasma kinin levels were elevated about 10–12 days after infection and at the same time there was a sharp decrease in kininogen levels [55–58]. Urinary kinin also increased in these rabbits [59]. Similar observations were made in two cattle infected with *T. brucei*. In this experiment, immunological studies were also undertaken measuring two variant antibodies by agglutination and neutralisation tests and two common antibodies using precipitation and fluorescent antibody methods [57]. Maximum kinin release occurred 10–12 days after infection just after the first antigenic variant was produced. Since there appeared to be a correlation between the peak of parasitaemia and kinin release, it was suggested that possibly the immune complex between trypanosome antigen and antibody which probably produced the fall in parasitaemia at this time also was involved in kinin release.

Corresponding falls in kininogen in the plasma of these cattle were noted. Of great interest was the observation that on treatment with the trypanocidal drug diminazene aceturate (Berenil, Hoeschst), a sudden increase in the plasma kininogen level occurred to approximately twice control levels, suggesting that during the infection the turnover rate of the

$\alpha_2$-plasma protein, kininogen, had been greatly enhanced [57]. In a human volunteer infected with *T. rhodesiense*, similar results were found with an increase in plasma kinin and corresponding decrease in kininogen. Again, release appeared to be associated with complex formation [60].

Another important feature of kinin release is the fact that in rats infected with the non-pathogenic organism *T. lewisi*, no kinin was detected [59]. In this infection, the number of parasites in the blood increase up to about day 10 when a trypanocidal antibody removes most of the parasites from the blood [61]; low levels of parasites remain until about day 60 when the animal becomes aparasitaemic. One would expect that conditions suitable for kinin release would exist, i.e. formation of immune complexes but this does not appear to occur. One possible explanation could be that the complex of *T. lewisi* and antibody is not negatively charged and thus cannot activate Hageman factor. Activation of Hageman factor is believed to be important in kinin release in trypanosome infections (see below). Activation can be blocked by positively-charged molecules [62] and a correlation exists between the degree of negativity expressed on the surface of the substance and its ability to induce activation [63]. Clearly this point requires further study since it could be important in understanding the pathogenic mechanisms of these parasites.

In vitro studies have been undertaken to try to elucidate the mechanism of kinin release [64]. Trypanosomes contained no free kinin or substrate for salivary kallikrein, nor were any kinin-forming enzymes detected. However, disintegrated but not whole trypanosomes did possess slight kininase activity but no evidence could be found for the presence in trypanosomes of inhibitors of bradykinin breakdown by plasma or erythrocyte kininases. The presence of kininase activity in trypanosomes is not surprising since many cells are known to possess this activity [65–67]. Since there appeared to be nothing inside the trypanosomes which accounted for the release of kinins, studies were undertaken on plasma kininase and kininogenase activity. Kininase activity of control and infected rat plasma was unchanged but the kininogenase activity fell from $38.0 \pm 5.4$ ng/ml bradykinin equivalents to $9.5 \pm 1.0$ ng/ml BK.eq. The prekininogenase levels also dropped from $223.7 \pm 24.9$ ng/ml BK. eq. to $110 \pm 10.2$ ng/ml BK.eq. Thus evidence had been produced for the activation of prekininogenase during trypanosome infections. It was considered that the most likely method of activation would be via Hageman factor which is known to be one method by which the kinin system may be activated; for a review see [68]. Particles with a negative

charge such as glass, kaolin and celite activate Hageman factor [69, 70] and so experiments were designed to see if immune complexes containing trypanosomes had the same effect [71].

Trypanosomes were separated from heavily-infected rat blood on DEAE-cellulose [72] and incubated with immune rabbit serum for 30 min at 37°C. The immune complexes were washed thoroughly and incubated with normal rabbit plasma in the presence of phenanthroline hydrate (100 $\mu$g/ml) in order to inhibit any kininases present. Samples were removed from the incubation mixture at intervals and assayed on the isolated rat uterus preparation. *Figure 2.3* shows that small amounts of kinin were released and the amount was dependent upon the number of parasites present in the complex. Trypanosomes alone or trypanosomes incubated in non-immune serum did not cause any kinin release. Heating the substrate plasma at 65°C for 15 min which destroys Hageman factor [73] prevented kinin release but heating the plasma at 56°C for 30 min had no effect. Thus complement was not involved in the kinin release.

The hypothesis is thus proposed that immune complexes formed during the infection absorb Hageman factor onto their surface causing its activation. Activated Hageman factor is known to have three effects [68]:

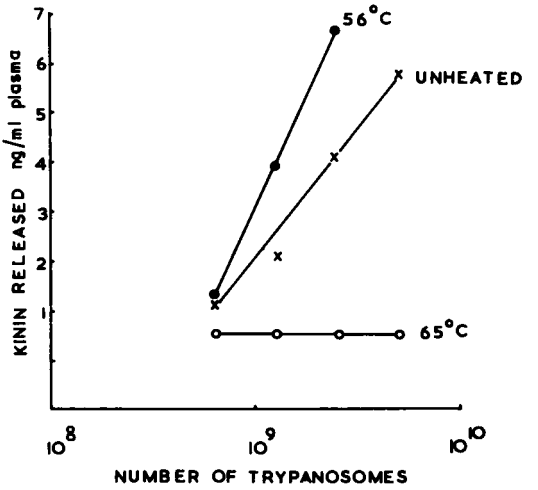


*Figure 2.3. Kinin release from kininogen substrate in the presence of a kininase inhibitor, phenanthroline, by immune complexes of trypanosomes and antibody. The diagram shows the effect of heating the substrate to 56°C and 65°C [72]*

(1) Activating prekallikrein to kallikrein resulting in kinin release from kininogen.
(2) Activating factor XI of the clotting system.
(3) Activating plasminogen activator which in turn converts plasminogen to plasmin.

The first of these mechanisms is suggested in trypanosomiasis and we might expect to find changes in the clotting and fibrinolytic systems as well. Studies on factor XI have not been undertaken but plasminogen activation does occur and this has been implicated in the release of fibrin/fibrinogen degradation products (FDP) [74, 75]. It is worth noting that FDP also show biological activity especially causing increased vascular permeability and potentiating biogenic amines [76].

Further evidence that immune complexes cause the release of pharmacologically active substances in trypanosome infections is given by the Schultz–Dale reaction. If whole separated trypanosomes are added to a tissue bath containing a piece of intestine from a non-infected animal no response is seen. However, if the intestine is taken from an animal previously infected with trypanosomes a slow contraction of the tissue occurs indicating release of contracting substances (*Figure 2.4*) [58]. In the case of rabbit intestine, this is largely 5-hydroxytryptamine and for guinea pigs histamine. The mechanism presumably is that trypanosome antigen combines with antibody in the gut wall causing the release of these pharmacologically active agents which in turn cause the contraction of the muscle.

Two other species of trypanosomes have also been studied. In a study of acute *T. evansi* infection in the guinea-pig [77], peptides, probably bradykinin, were shown to be released.

The mechanism of kinin release by immune complexes does not explain the presence of these substances in acute infections where little antibody is present. Possibly direct tissue damage and activation of kinin-forming enzymes is involved. In a very recent study [78] of goat infected with *T vivax* bradykinin-like activity was found very early in the infection before complexes would be found. Veenendaal, van Miert, van den Ingh, Schotman and Zwart [78] suggest that the kinin release may be associated with pyrexia and be similar to endotoxin shock [79].

### *Malaria*

In the reports of kinin release in malaria infections, the terms bradykinin and bradykininogen are widely used when it has not been shown to the

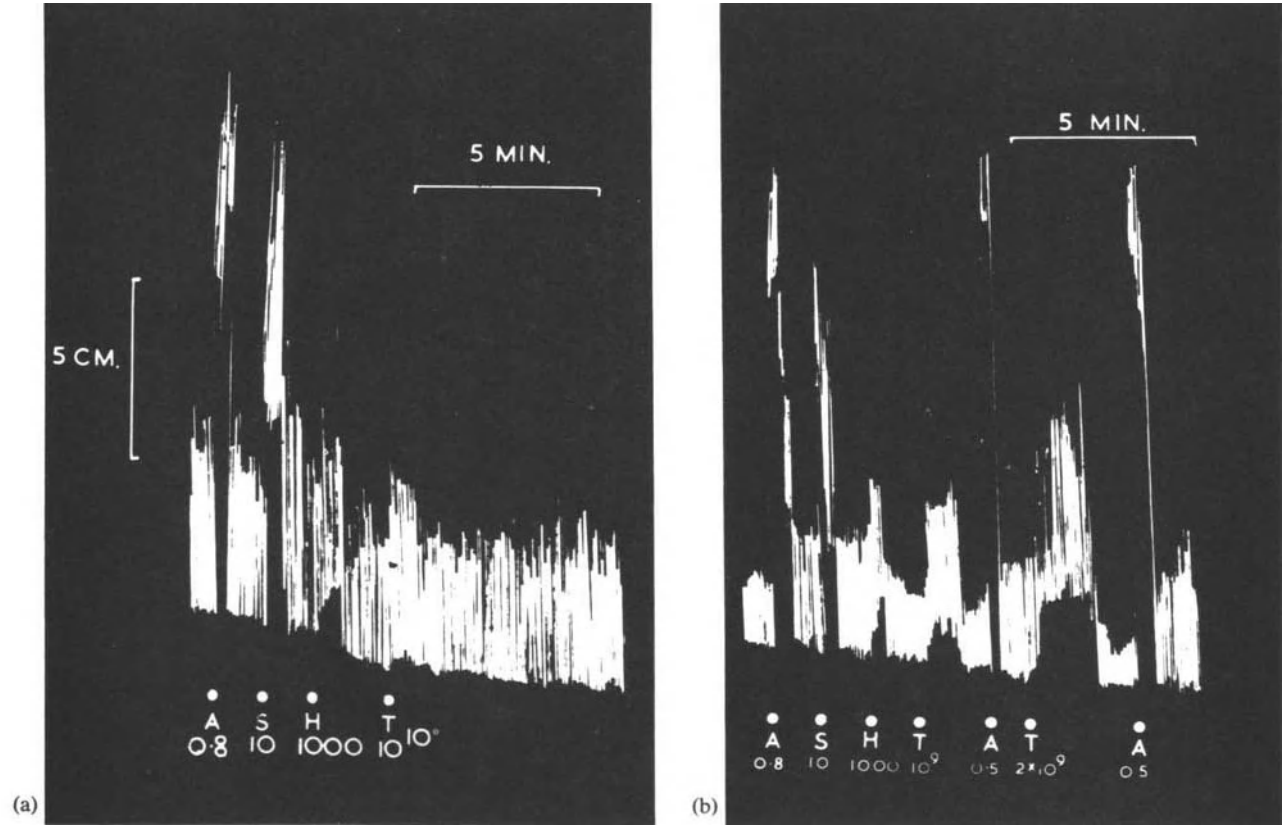


*Figure 2.4. Effect of trypanosomes on the isolated rabbit ileum. (a) Non-infected rabbit ileum. (b) Ileum from rabbit infected with* Trypanosoma brucei. *Drug doses are expressed in μg/ml (A) Acetylcholine; (S) 5-hydroxytryptamine; (H) histamine, (T) washed separated trypanosomes [58]. Note on infected tissue after adding trypanosomes the response to acetylcholine is potentiated*

reviewers' satisfaction that this is the actual substance being measured. Therefore, the terms kinin and kininogen are preferred.

Plasma kinin and kininogen levels were studied in acute infections of monkeys with *Plasmodium knowlesi* [80–87]. A marked reduction in kininogen occurred as the infection progressed and only 8% of the original concentration remained on day 5 just prior to death [83]. This fall appears not to be due to decreased kininogen production since the $\alpha$-globulin levels were raised 60% just before death occurred. Thus the fall in kininogen was assumed to be due to increased utilisation. No rise in plasma kinin levels was detected [85, 86] although a decrease in precursor was found. It was postulated that elevated kininase activity prevented measurement of free kinin. However, in a later study [87] free kinin was found in the blood of acutely infected monkeys. The kinin concentrations in the urine have also been shown to increase [88].

One study has been undertaken on kinin and kininogen concentrations in *P. berghei* infections of mice [89]. In severe infections the plasma kininogen level fell from 8.92 $\mu$g/ml to 6.00 $\mu$g/ml but in mild infections there appeared to be no fall. The results are expressed in terms of $\mu$g of kininogen per mg of globulin. For control mice it was 0.376 $\mu$g/ml globulin in mild infections 0.362 $\mu$g/ml globulin and for severe infections 0.259 $\mu$g/ml globulin. In this report, criteria are not given for classification of the severity of the infections.

In both rodent and monkey malarias, plasma kininase activity increases [89, 90]. In *P. knowlesi* infections a 3–4-fold increase was noted whereas in both mild and severe *P. berghei* infections a 2–3-fold increase was found.

The direct involvement of kallikrein in *P. knowlesi* malaria has also been shown [85, 86, 91–93]. Kallikrein levels increased by 50% during the late stages of an acute infection. This rise was accompanied by a corresponding fall in the kininogen, suggesting a large turnover of kinins. Kallikrein activity was found in all five chromatographic peaks obtained from DEAE-cellulose chromatography though most was associated with the $\gamma$-globulins [93]. No explanation was offered for the elevated kallikrein levels and the mechanism of kinin release remains uncertain.

Following the demonstration of raised kallikrein levels attempts were made to assess the physiopathological importance of this enzyme. The extract obtained from the DEAE-cellulose column increased capillary permeability on intradermal injection into guinea pigs and rabbits previously injected intravenously with potamine sky blue. After about one hour the dermis of the skin was infiltrated with leucocytes consisting

mainly of lymphocytes and eosinophils [85]. These changes are similar to those seen in the small vessels of the brain in malaria and so it was suggested that kallikrein might, at least in part, be responsible. Further support for this was obtained by injecting kallikrein extracts of monkey plasma intracerebrally into 'blued' normal guinea pigs. Widespread disturbances of the blood–brain barrier were noted, resulting in high molecular weight substances, such as albumin, passing into the perivascular spaces of the brain tissue and so to the cerebrospinal fluid. Activity was present in normal and infected plasma but greater in the latter case. Intravenous injections of the kallikrein fractions caused a significant fall in arterial blood pressure when injected intravenously into rabbits [85, 93]. It appears therefore that kallikrein released in the blood during *P. knowlesi* infections of monkeys could lead to the development of inflammatory stasis in the small vessels of the brain.

It must be remembered that all the work on the kinin system in malaria relates to acute infections and great care must be taken in extrapolating to man. Measurement of some of the components of the kinin system in human infections is certainly required.

### *Babesia*

In *Babesia* infections of cattle, extensive studies have been undertaken on the role of kallikrein but to date kinin and kininogen concentrations have not been measured. Kallikrein was assayed by means of its esterolytic activity using $N$-$\alpha$-tosyl-L arginine methyl ester (TAME) as the substrate. The biological activity of the samples was determined using the guinea pig permeability method and measuring the amount of Evans blue dye leaking out of the circulation after intradermal injections of the enzyme [94]. Kallikrein activity rose markedly three days after infection and fell to subnormal levels terminally. In mildly infected calves, the level fell slightly on day 3 and remained stationary thereafter. Most of the kallikrein activity was associated with $\beta$- and $\gamma$-globulins. Activation of prekallikrein to kallikrein commenced in acute *B. argentina* infections of calves 1–2 days before parasites were detectable in peripheral blood (when an estimated $10^9$–$10^{10}$ parasites were present) and continued until prekallikrein levels were less than 10% of normal values, 11–12 days after infection [95]. The total kallikrein level (prekallikrein and kallikrein) rose by up to 50% six days after infection but fell to about 50–60% of normal terminally. This work suggested that there is massive mobilisation and activation of kallikrein during acute *B. argentina* infections. The mechan-

ism of activation was considered to be one of parasite secretion for $10^{12}$ lysed normal red cells injected into control animals failed to activate kallikrein. This control experiment ruled out the involvement of tissue products as activating substances at least early in infection as only an estimated $10^9$–$10^{10}$ red cells are parasitised when activation begins.

The in vitro activation of plasma prekallikrein by purified parasite esterases has been shown to occur [96]. As much of 90% activation occurred after 1 h incubation, at 37°C, of normal plasma with the parasite esterase. Concurrent coagulation of plasma occurred. The mechanism of activation was considered to be direct for prekallikrein, but since calcium ions were absent from the plasma the concurrent coagulation was thought not to be due to the direct activation Hageman factor.

The in vivo activation of plasma prekallikrein has also been demonstrated in cattle by Mahoney and Wright [97]. They showed that within 30 min of intravenous injection of $10^8$ lysed parasites in 2.5 ml red cell stroma there was a 150–200% increase in activated kallikrein levels and a 25–30% fall in the packed cell volume. These parameters returned to normal after 24 h. The intravenous injection of 2.5 ml isologous red cell stroma into control animals had no effect.

In another study [98], the broad spectrum proteinase inhibitor Trasylol (2000 kallikrein inhibitor units per kg body weight) was administered to a group of calves infected with *B. argentina* for five days commencing on the third day of the infection. A second group was treated with the same dose for three days, commencing 8 days after infection at which time the animals were in severe shock. A third group of infected calves were retained as a control group. Parasite multiplication rates were similar in the three groups. In the group treated early, levels of activated kallikrein in plasma were significantly lower than those in the other two groups. Packed cell volumes were also significantly higher in both treated groups. This study strongly supported the idea that plasma kallikrein is important in the pathogenesis of acute babesiosis. In addition, the higher packed cell volumes in treated calves indicated that much of the red cell loss is apparent only, and is due to vascular congestion, for parasite multiplication rates, and presumably red cell penetration and destruction by parasites, were similar in the three groups.

To date, no investigations have been made on the possible importance of immune complexes in *Babesia* infections. However, since it is an acute infection killing cattle in about 8 days, it would seem unlikely that sufficient time is available for immune complexes to be a significant mechanism of kallikrein activation. Also, the in vivo activation of

kallikrein by disrupted parasites also precludes the role of immune complexes in this disease.

Thus, in the two infections studied in detail, chronic trypanosomiasis and acute babesiosis, two different mechanisms of kallikrein activation occur. In the first instance, it appears that immune complexes activate Hageman factor and in the latter case direct activation of prekallikrein occurs by parasite esterases. Since *P. knowlesi* infections of monkeys are also acute a similar mechanism to *Babesia* infection may be present.

### THE KININ SYSTEM IN HELMINTH INFECTIONS

Only one report exists on studies of the kinin system in helminth infections [99]. In rats infected with 2000 larvae of *Trichinella spiralis* plasma kininogen levels fell from about 4.5 $\mu$g/ml Bk.eq. to just over 1 $\mu$g/ml BK.eq. No attempt was made to measure free kinin or kininogenases. Lowest levels of kininogen were found 15–25 days after infection whereas maximum mast cell counts in skeletal muscle and biogenic amines were found 30–35 days after infection.

## RELEASE OF PHARMACOLOGICALLY ACTIVE SUBSTANCES IN ANAPHYLAXIS

Histamine was the first chemical to be implicated in anaphylaxis [100, 101] but direct evidence for its release in vivo and in vitro in anaphylaxis of guinea-pigs was not obtained until 1932 [102, 103]. Subsequently, numerous studies have been undertaken and the four main chemicals which have been shown to be released are histamine, 5-hydroxytryptamine (5-HT) [104, 105], slow reacting substance of anaphylaxis (SRS-A) [106] and kinins [107]. The importance of these chemicals differs for different host species. In laboratory studies of parasites, hosts such as guinea-pigs, rats and rabbits are commonly used. It is known that in the rat, 5-HT and histamine play little part in anaphylaxis, in vivo, since shock will still occur in animals when histamine and 5-HT levels have been depleted or specifically inhibited by antagonists. In the guinea-pig, however, both histamine and 5-HT are involved in anaphylaxis [108]. Studies of the distribution of 5-HT and histamine in rats and guinea-pigs have shown that the skin of rats contained about 50% of the total 5-HT in this animal and that it is associated with mast cells. In the guinea-pig, 5-HT is not associated with mast cells [109, 110].

Table 2.4. 5-HT AND HISTAMINE CONCENTRATIONS IN THE TISSUES OF THE RAT AND GUINEA-PIG [109]

| | *5-HT (μg/g)* | | *Histamine (μg/g)* | |
|---|---|---|---|---|
| *Tissue* | *Rat* | *Guinea-pig* | *Rat* | *Guinea-pig* |
| Ears | 1.01 | 1.18 | 41 | 12 |
| Spleen | 2.5 | 1.1 | 2 | 1 |
| Lungs | 1.16 | 0.06 | 9 | 40 |
| Liver | 0.14 | 0.06 | 1 | 6 |
| Abdominal skin | 1.34 | 0.02 | 23 | 3 |
| outer layer | 1.54 | 0.04 | 13 | 2 |
| inner layer | 0.92 | 0.08 | 32 | 3 |

*Table 2.4* taken from data obtained by Parratt and West [109] details the distribution of these two amines in the guinea-pig and rat.

These figures also illustrate the fact that the 'shock organ' varies with different host species. In the guinea-pig, the lung and bronchioles are the primary shock organ whereas in rats it is the circulatory system and gut [111].

In the rabbit, there is evidence that all four pharmacologically active substances may be involved but in the mouse histamine appears to be unimportant and the main mediator is probably 5-HT [112].

In calves, histamine and 5-HT were at first thought to be important mediators of anaphylaxis [113], but subsequently the use of sodium meclofenamate and diethylcarbamazine, compounds which block kinins and pharmacologically active lipids (SRS-A and prostaglandins) indicated that kinins and SRS-A are much more important than amines in calf anaphylaxis [114–116].

More recently another group of workers [117] concluded that SRS-A, histamine, 5-HT and possibly other substances act synergistically in small doses to produce anaphylaxis in calves.

When an antigen is administered to sensitised animals, histamine is released [106]. However, although histamine plays some part in the antigen–antibody reactions, the release of histamine does not appear to be the immediate consequence of the antigen–antibody combination [118]. Histamine levels in tissues have been correlated with mast cell numbers by many authors, see [119, 120] and at least half the total histamine concentration of normal blood is found in basophils, despite their scarcity [121]. Mast cell degranulation with the associated release of vasoactive amines and SRS-A has been reviewed by Stanworth [122].

Antigen–antibody reactions caused by hypersensitivity induce an

eosinophilia [123], as does the repeated injection of foreign proteins [124] or histamine [125]. Eosinophils are found in areas where mast cell granules, and presumably histamine is being released [126], although some workers have been unable to show this [127]. However, it appears that the relationship between eosinophils and histamine may be due to the fact that eosinophils contain an antihistamine [127] and an arylsulphatase which is thought to destroy SRS-A [128]. A factor has been isolated from cell-free extracts of anaphylactic lungs which caused eosinophil production [129]. It has also been demonstrated that cortisone, which reduces inflammatory cell infiltration, also impairs mast cell function [130].

## HISTAMINE AND 5-HYDROXYTRYPTAMINE RELEASE IN PARASITIC INFECTIONS

Since histamine and 5-HT are often released together during anaphylactic reactions, these two compounds will be considered together.

The pharmacology of histamine and 5-HT have been extensively documented and fall outside the scope of this review. The interested reader is referred to the published symposia and monographs [131–135].

Most of the studies on histamine and 5-HT in infections concern their possible importance in helminth infections rather than protozoal infections. Many authors have implied that histamine is released because of the eosinophilia associated with parasitic infections. In this review we have confined our comments mainly to those situations in which histamine and 5-HT levels have actually been measured.

### PROTOZOAN INFECTIONS

Major studies of histamine concentrations have been undertaken in only two acute infections, *T. brucei* of rodents and *P. knowlesi* of monkeys. A 2–3-fold increase in tissue histamine was seen 2–3 days after infection of mice with *T. brucei* but by the fifth day prior to death, the level had started to fall [53]. Plasma histamine levels rose from 130 ng/ml in control mice to maximum levels of 190 ng/ml on day 3 and had fallen to 150 ng/ml by day 5. These studies have been continued by Yates [136, 137] who used rats as the host. During acute *T. brucei* infections he was unable to find any change in either histamine concentration or in the histamine-forming enzyme histidine decarboxylase levels in blood and tissues. The role of histamine in acute trypanosomiasis is still unresolved but the fact that

different hosts have been used may be a significant factor. However, it is of interest to note that mast cell degranulation has not been observed in *T. brucei* infections of the mouse [55].

In acute *P. knowlesi* infections of monkeys, blood levels of 150 ng/ml histamine have been reported whereas in the non-infected monkey histamine was not detected [138, 139]. It was suggested that this release may be due to the action of phospholipase *A* of red cells although there is no evidence to support this suggestion. The histamine extract from infected monkey plasma was shown to cause increased vascular permeability in the skin and brain vessels of guinea pigs and also to produce hypotension when injected intravenously into rabbits.

In *B. rodhaini* infections of mice, histamine release was observed in similar amounts to that of *T. brucei* infections of the same host, see [151]. The mechanism of release was not investigated.

The only published report on 5-HT in protozoal infections concerns *T. vivax* in the goat [78]. During this infection, it was found that there was a gradual decrease in blood 5-HT concentrations which returned to normal as the infection progressed. 5-HT concentrations in the blood fluctuated inversely with temperature. Changes in the amounts of 5-HT may be related to the formation of platelet thrombi, an important pathological finding in this disease.

## HELMINTH INFECTIONS

The release of vasoactive amines during helminth infections has been demonstrated by numerous workers. In most instances, the release of amines appears to be related intrinsically with the 'self-cure' and other immune phenomena. The actual relationship of amines to worm expulsion has been established by several workers, but much controversy still exists as to their function in the expulsion process.

Much of the work relating amines to worm expulsion has been based on circumstantial evidence obtained from the administration to the host of the amines themselves, specific amine antagonists or mast cell counts.

### *The 'self-cure' phenomenon*

'Self-cure' was first described in *Haemonchus contortus* infections of sheep [140]. Worm-free lambs were infected with *H. contortus* and were then turned out to graze on a limited pasture where they were subject to re-infection. Sudden and dramatic falls in egg counts were observed which were thought to be due to the elimination of adult worms. The

'self-cure' phenomenon was actively studied in the 1950s by Stewart [141–145].

Stewart [144, 145] showed that in sheep infected with and suitably sensitized by *H. contortus*, a challenge dose of *H. contortus* infective larvae would cause a sudden and dramatic loss of adult worms. Blood histamine levels rose at the same time as the complement-fixing antibody titres increased. He explained this as the effect of an antibody–cell complex and the subsequent discharge of granules from mast cells by the appearance of antigen. The pharmacologically active substances released probably caused an increased gastro-intestinal motility and marked oedema, as a result of the expulsion of adult worms. In parasitised animals a rise in blood histamine occurs when third-stage *H. contortus* larvae moult to the fourth-stage larva [146, 147]. The antigenic stimulus probably results from the exsheathing fluid released by larvae during moulting.

He concluded that:

(1) The reaction was precipitated in an animal harbouring a population of adult worms by the intake of a new dose of infective larvae.
(2) The reaction did not occur in all infected sheep challenged by larvae, but was most likely to occur in animals which had had several previous infections with *H. contortus* and were showing good 'immediate-type' skin reaction to *Haemonchus* antigen.
(3) In some cases, the adult worm population was largely eliminated, in others the 'self-cure' was only partial in that only a proportion of worms was expelled, while in others all that resulted was a transient drop in egg production.
(4) The reaction was not entirely specific. For example, when sheep infected with both *H. contortus* and *Ostertagia* sp. were challenged with *H. contortus* larvae, adults of both species were eliminated. Likewise in mixed infections of *H. contortus* and *T. colubriformis* the latter parasite was also eliminated from the small intestine during the 'self-cure' reaction.

'Self-cure' in *T. colubriformis* and *Ostertagia* sp. infections is similar to that described above for *H. contortus*. However, in both *Trichostrongylus retortaeformis* infections of the rabbit [148] and *Nippostrongylus brasiliensis* infections in the rat [149], a sudden termination of a primary infection occurs which is not associated with a fresh uptake of larvae. This has been attributed to a local anaphylactic reaction caused by the production of an allergen by the adult worm. The 'self-cure' phenomenon in *H. contortus* and *T. colubriformis* is believed to be associated with a

local anaphylactic reaction caused by the larval worm [144, 146, 147, 149] during the third ecdysis [147].

The role of local anaphylaxis in worm expulsion was studied by the pretreatment of recipient rats with ovalbumin [150]. *N. brasiliensis* worms were then transplanted into these animals, which also received hyperimmune serum prior to a shocking dose of ovalbumin. The administration of the shocking dose of ovalbumin caused an accelerated worm loss when compared with identical animals which either received hyperimmune serum alone, or were shocked and did not receive hyperimmune serum. A two-stage mechanism was therefore proposed. Initially, reaginic antibody induces hypersensitivity in the immune rats and this is followed by the release of vasoactive amines which assist in the translocation of anti-worm antibody to parasitised sites [151].

The mechanism of 'self-cure' in *N. brasiliensis* infections in the rat has also been investigated [149, 150] and it was suggested that: (i) antibody acts directly on the worm, and (ii) the antigen–antibody response causes a local anaphylactic reaction with increased vascular permeability and significant extravascular leakage of plasma.

The 'leak lesion' hypothesis has also been postulated to explain worm expulsion [152]. This theory suggests that amine release causes an increase in the permeability of the intestinal epithelium, which in turn results in an increased concentration of anti-worm antibody around the worm. This increased concentration of anti-worm antibodies was thought to be directly responsible for worm expulsion. It seems probable, though, that anti-worm antibody acts in conjunction with other components in the immune system, suggesting that this theory is only part of the explanation of why worms are rejected from the gut.

Ogilvie and Jones [152] state that 'the release of amines from mast cells can be triggered either by the interaction of reagenic antibodies and worm allergen, or by the action of a degranulator produced by worms which does not require the co-operation of antibody for its action. The existence of the latter method of amine release explains why the presence of reagenic antibody is not essential for worm expulsion to occur in passive immunity. In active immunity, the former method of amine release from mast cells is probably the more effective trigger.'

### *Mast cells and eosinophils in helminth infections*

Circumstantial evidence for the role of amines in the expulsion of worms has come from studies on the involvement of myeloid cells in the

rejection process. Although these studies are too numerous to list fully, we have endeavoured to include major references in which degranulation of mast cells has been observed in relation to worm invasion or expulsion. Mast cell granules contain the vasoactive amines, histamine and 5-HT [153] which are discharged into the surrounding tissue by the stimulus of antigen–antibody reaction and by chemicals such as compound 48/80 [154]. Mast cell membranes have a strong affinity for globulins and in particular IgE (reagenic antibody) [155].

The involvement of eosinophils and mast cells in the 'self-cure' phenomenon of helminthiasis was first reported in 1939 [156] when it was shown that mast cell numbers increased in the intestinal mucosa of rats infected with *N. brasiliensis*, during the process of worm expulsion. Subsequently it was shown that there was also an eosinophilia and it appears that mast cell numbers only rise after worm expulsion [157]. Examination of the mucosa of 'self-cure' sheep previously infected with *H. contortus* indicated that there was a marked oedematous change with superficial aggregation of eosinophils suggestive of a hypersensitivity state [158]. In *A. suum* infections in pigs, changes in the intestinal mucosa including eosinophilia, oedema and vasodilation suggest that the changes might be due to the release of pharmacologically active substances from mast cells [159].

Several studies on mast cells have been undertaken in rats infected with *N. brasiliensis*. Numbers in the intestinal wall increase during worm expulsion and there is a corresponding increase in the concentration of 5-HT at the site of maximum worm concentration [160]. Several workers have demonstrated that amines are released from mast cells at this time [161–164]. It appears that during primary infection with *N. brasiliensis* degranulation of mast cells occurs just after the young adult worms reach the intestine following their migration [165]. However, there is evidence that degranulation of mast cells also occurs in the lung while the worm is migrating. This is either in response to stress or to the release of degranulating substances by the larva [166].

Mast cell studies have also been undertaken in *T. colubriformis* infections of the guinea-pig [167], *Apatemon gracilis* a trematode parasitising the gut of ducks [168], *T. spiralis* in mice [169, 170] and *Strongyloides ratti* in mice [171]. It appears that mast cells play no part in infection of the larval cestode *Taenia taeniaformis* of rats [172].

There are two mechanisms by which mast cell degranulation could occur: (i) directly by substances of parasitic origin, (ii) by reagenic antibody. Both *Ascaris suum* and *N. brasiliensis* contain allergens and

substances which degranulate mast cells [173–180]. Reaginic antibody IgE is produced in many helminth infections including cestodes, nematodes and trematodes [152, 155]. This combines with antigen-sensitised mast cells causing degranulation and such a mechanism may be important in the 'self-cure' reaction.

*Amine levels in helminth infections*

The release of pharmacologically active amines during the expulsion of *H. contortus* [144–147] have led to numerous studies which have demonstrated elevated vasoactive amine levels in primary helminth infections.

Wells [157] has carried out an extensive study on histamine concentrations in the tissues of rats infected with *N. brasiliensis* and fed two different diets. The results were correlated with mast cell and eosinophil numbers. A summary of her results is shown in *Table 2.5*. The decrease in mast cell numbers up to day 16 was explained by the release of histamine which in turn caused an eosinophilia from day 12 onwards. When these rats were challenged with the same infection at 34 days i.e. after 'self-cure' had occurred, a significant fall in mast cell numbers was noted and a concurrent rise in histamine concentration occurred 5 days later. Eosinophils did not respond and it was suggested that this was due to exhaustion of the system. Rapid expulsion of worms occurred at this time. In the rats fed on a low protein diet mast cell numbers and histamine levels were below normal 5 days after challenge infection. There were fewer mast cells and their disruption was delayed. It was concluded that this delay must have affected the 'self-cure' phenomenon to *N. brasiliensis* for worm expulsion only occurred after histamine levels rose on day 12 [157]. Lowered histamine levels in the small intestinal mucosa of rats undergoing primary infection with *N. brasiliensis* have also been reported [181]. Again in this study the histamine levels rose to slightly higher than normal levels after worm expulsion. Another group [160] has shown that 5-HT levels in the small intestinal mucosa fall to below normal levels after primary infection of rats with *N. brasiliensis* and then rise above normal after worm expulsion. Antigen-induced amine release from mast cells as early as 9 days after infection of rats with *N. brasiliensis* has also been described [182]. This release of amines coincided with the initiation of tissue damage by the worms. However, Keller was unable to confirm this with *N. brasiliensis*-infected rats [183].

Pharmacologically active amines have also been studied during primary and challenge infections in *T. colubriformis* infections of guinea-pigs.

Table 2.5. HISTAMINE, MAST CELL AND EOSINOPHILS IN RATS INFECTED WITH 5000 LARVAE OF *N. BRASILIENSIS* [157]

| | Days after infection | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 5 | 9 | 12 | 16 | 23 |
| (A) Rats fed on normal diet | | | | | | |
| Tissue histamine ($\mu$g/g) | 12.1 ± 1.1 | 1.1 ± 0.3 | 15.0 ± 7.3 | 14.5 ± 0.3 | 14.6 ± 9.0 | 15.7 ± 11.4 |
| Mast cells[a] | 29.6 ± 11.4 | 11.2 ± 2.5 | 5.9 ± 2.7 | 7.2 ± 2.7 | 6.3 ± 5.1 | 83.1 ± 4.9 |
| Eosinophils[a] | 28.1 ± 9.5 | 51.9 ± 14.5 | 71.4 ± 22.2 | 71.3 ± 1.7 | 77.5 ± 3.8 | 76.9 ± 9.7 |
| Worm burden[b] | – | + + + | + + + | + + | – | – |
| (B) Rats fed on low protein diet | | | | | | |
| Tissue histamine ($\mu$g/g) | 20.6 ± 11.0 | 5.3 ± 1.6 | 3.1 ± 2.2 | 6.5 ± 2.6 | 16.7 ± 5.7 | 77.8 ± 16.7 |
| Mast cells[a] | 11.3 ± 3.6 | 17.2 ± 9.9 | 5.8 ± 1.4 | 0.8 ± 0.15 | 24.9 ± 10.1 | 38.3 ± 8.6 |
| Eosinophils[a] | 30.3 ± 3.7 | 54.0 ± 7.1 | 38.0 ± 10.0 | 11.0 ± 0.15 | 33.0 ± 2.4 | 64.0 ± 9.8 |
| Worm burden[b] | – | + + + | + + + | + + + | + + + | + or – |

[a]Cells per 20 fields at × 800

[b]Numbers estimated by eye: + + + maximum number of worms. – no worms present.

The histamine content of the small intestinal mucosa during primary infection of guinea-pigs [184] increased from 15.7 μg/g mucosa in control animals to 50.3 μg/g on day 20 and 58.8 μg/g in animals after more than 50 days infection. Similarly, a 3-fold increase in 5-HT concentration was seen in the intestinal mucosa but this was more erratic, a fall being noted about the time worm expulsion occurred. The levels of amines rose too early (day 5) during primary infection to be attributable to an immune response and so it was suggested that amines were recruited into the mucosa by a stimulus resulting from the infection. The reduced 5-HT levels which corresponded with worm expulsion are consistent with the view [167] that an immunological trigger causes mast cell degranulation, amine release and therefore a temporary depletion of tissue amine stores. When guinea-pigs previously infected with *T. colubriformis* were challenged with the same worm an additional increase in histamine in the mucosa and a transient decrease in 5-HT levels was observed. The fall in 5-HT was attributed to an accelerated release due to an enhanced immunological response and was correlated with the period of worm expulsion. In contrast, mucosal histamine levels reached a peak during the period of worm expulsion. It was concluded that amines are released during worm expulsion, a finding strongly supported by the presence of higher levels of both histamine and 5-HT in the intestinal contents of challenged immune animals than in the intestinal contents of non-challenged immune, non-immune and challenged non-immune animals.

The simultaneous infusion of both histamine and 5-HT into the small intestine of guinea-pigs infected with *T. colubriformis* during the fourth larval stage (day 5–9) led to expulsion of a significant number of worms [185]. However, if either amine is administered alone during this period, or if both were administered during the first seven days of infection (third stage and early fourth stage larvae) or on days 8–12 (late fourth stage larvae and early adult), the treatment was ineffective. These results suggest that for worm expulsion, amines need to be present in high concentrations throughout the fourth larval stage. The incubation of fourth stage larvae for 8 h with amine solutions in vitro did not inhibit the ability of these worms to parasitise a new host [185]. This finding is also consistent with the theory that amines need to be present throughout the fourth larval stage for worm expulsion to occur.

Elevated levels of 5-HT, histamine and mast cells have been reported in the intestinal mucosa of rats 5–25 days after infection with *Trichinella spiralis* [99]. This increase takes place only when fertilized *T. spiralis* females bear live larvae in the intestine which enter the blood stream and

migrate to skeletal muscle. This rise is followed by a rapid decrease in mast cell numbers and amine levels, concurrent with violent expulsion of parasites from the intestinal tract. Mast cell counts and histamine level rose in skeletal muscle 25–35 days after infection coinciding with the peak of muscular invasion which occurred 4–5 weeks after infection. Reduced lung, brain and kidney tissue levels of histamine have been reported 6 days after *Ascaris suum* infection of guinea pigs and at the same time histaminase activity of liver was increased [186].

In order to determine whether the release of these pharmacologically active substances has any effect on the host, studies have been undertaken in rats on the permeability of the gut wall during *N. brasiliensis* infection [161, 165, 187]. In all cases increased permeability was found. During the phase of worm loss, i.e. days 11–16 in the primary infection, the permeability of the wall of the large intestine to macromolecules is increased and this was especially marked on day 14 at the time of the onset of the rapid phase of worm expulsion [188]. Administration of cortisone, an anti-inflammatory drug, suppressed this reaction.

*Use of amine inhibitors*

The role of histamine in *N. brasiliensis* expulsion in the rat was indicated by using the antihistamine and anti-5-HT drug promethazine [189]. This drug was shown to inhibit worm expulsion. However administration of either an antihistamine drug or an inhibitor of 5-HT alone had no effect. In another study on the role of pharmacologically active amines in *N. brasiliensis* expulsion in the rat, inhibitors of histamine (promethazine hydrochloride) and 5-HT (501C67) were administered daily [190]. These drugs prevented the onset of the rapid phase of worm expulsion, but did not stop the fall in egg production which always precedes worm loss. It was suggested that availability of anti-worm antibody (which presumably depresses egg production but not worm expulsion) was not impeded by these inhibitors. A further study on the role of promethazine in *N. brasiliensis* expulsion suggested that this drug may inhibit resistance to the parasite by immunosuppressive action at the lymphoid level either in addition to, or instead of, antagonism of amines [191]. The administration of reserpine (which depletes tissue stores of amines) to *N. brasiliensis*-infected rats also inhibits worm expulsion [192]. The intraduodenal injection of either histamine dihydrochloride, or 5-HT on day 6 of the primary infection of rats with *N. brasiliensis* however failed to cause worm expulsion [193]. Similarly daily administration of low doses of

histamine (15 mg/kg) failed to produce worm expulsion. It was therefore concluded that histamine was not involved in the expulsion process of *N. brasiliensis* of rats [193]. Numerous other authors also argue against the role of amines in the expulsion of *N. brasiliensis* from the rat [157, 181, 183, 191, 194]. Although specific amine antagonists appear to prevent expulsion of *N. brasiliensis* in some studies, these drugs may in fact be acting in some other way than by direct antagonism [194, 195]. Furthermore, one group of workers considers that histamine levels in the intestinal mucosa are markedly reduced at the parasitised site throughout infection and remain low at this site even during worm expulsion when histamine levels in the remainder of the intestine are considerably increased [152].

Administration of the antihistamine chlorpheniramine and a 5-HT antagonist (benanserin hydrochloride, 1-benzyl-2-methyl-5-methoxy-tryptamine hydrochloride) in *T. spiralis* infections in mice also prolonged the intestinal phase of infection [196].

The problem with many of these studies is that very large doses of drugs have been used which will have other effects particularly on the immune system. Therefore, it is very difficult to interpret the results of such experiments.

The situation with *T. colubriformis* infections in guinea-pigs is more straightforward. There is almost universal agreement that amines are important in the expulsion of worms.

The administration of promethazine to guinea-pigs was found to suppress the development of resistance during primary infection and inhibited expulsion of the parasite in actively and adoptively immunized animals [197]. Mepyramine and the $\alpha$-hydrazino analogue of histidine (to inhibit histamine formation) both prevented expulsion of the parasite in actively immunized guinea-pigs, but methysergide (a 5-HT antagonist) and $\alpha$-methyl dopa (to inhibit 5-HT synthesis) were not effective. Reserpine was found to suppress rejection of a challenge infection in actively and adoptively immunized animals. The oral administration of the histamine precursor (L-histidine) and 5-HT increased the resistance during primary infection of non-immune animals [197]. It was concluded that both histamine and 5-HT are important in the resistance of the guinea-pig to *T. colubriformis*. A biphasic mechanism of resistance of animals to helminth infections was proposed.

The first phase of *T. colubriformis* rejection from the gut of guinea-pigs is immunologically specific and probably involves the interaction between antigens and sensitized lymphocytes, which acts as a

trigger for myeloid (eosinophil and basophil) involvement, and the release of pharmacologically active amines. The second phase, which is non-specific, appears to be the final effector mechanism, and involves the rejection of parasites either directly or indirectly by the action of the amines [197]. In another study an attempt was made to elucidate the mechanism of action of promethazine which prevents worms expulsion [198]. The effect of promethazine on the capacity of lymphoid cells from immune donors to transfer immunity to syngeneic recipients in *T. colubriformis* infections of guinea-pigs was studied. Some inhibitory action was observed and certainly the prevention of worm expulsion by the drug does not seem to be entirely due to its antagonism of amines. However, despite the reduction in the capacity of lymphoid cells from promethazine-treated immune guinea-pigs to transfer immunity, these cells were still able to transfer a high degree of immunity [198]. The expulsion of *T. colubriformis* from adoptively immunized guinea-pigs has also been inhibited by reserpine [199] which depletes tissues of 5-HT [200].

### *Anaphylaxis caused by helminth extracts*

Another aspect of the release of pharmacologically active substances in helminth infections which must be considered is the induction of anaphylaxis by injection of helminth extracts. Transfusion of blood containing *D. immitis* microfilariae into a non-infected dog produced an anaphylactic-like reaction [201, 202]. Likewise anaphylaxis has been reported in *S. vulgaris* infections of horses sensitised by previous infection [203]. Injection of extracts of worms into immune animals will also cause anaphylaxis. Numerous studies have been undertaken including *Ascaris* sp. *Toxocara canis, Trichinella spiralis* and *Paragonimus ohiri* [204–211].

### *Conclusions*

Although conflicting evidence exists, it appears that most authors are now in agreement that mast cell discharge and amine release play at least a minor role in worm expulsion. The expulsion of *N. brasiliensis* is thought to be due to a sudden release of amines from mast cells as a result of interaction of reaginic antibody on the mast cell surface with worm antigen [177, 187], but antibody and cells are also involved.

The situation with other gastrointestinal helminths and, in particular,

*H. contortus* and *T. colubriformis* is one of almost universal agreement that histamine and/or 5-HT are deeply involved in the worm expulsion process. Although histamine has been mainly implicated in *H. contortus* expulsion [144, 145], it appears that 5-HT is the main mediator of *T. colubriformis* expulsion [199]. This difference is probably related to the presence of these substances in the host.

## CATECHOLAMINES

Much is known about the role of catecholamines in bodily function and in stress situations [212] but, although shock-like syndromes are common in parasitic diseases, there is little direct evidence of the involvement of these substances. Involvement of sympathetic nervous mechanisms is implied from a number of studies.

The only parasitic disease where direct measurements of catecholamines have been undertaken is trypanosome infections. Since vascular collapse is one of the features of the disease [213], Goodwin postulated that failure of the adrenal medulla might be important.

No evidence of depletion of adrenaline was found in the adrenal glands of rats acutely infected with *T. brucei* nor in rabbits with a chronic infection [136, 137]. However, in the latter case, depletion of noradrenaline from the heart occurred. In order to investigate whether increased turnover of catecholamines occurred in trypanosome infections, the metabolites in the urine were examined [137]. The major metabolite is unknown in rabbits but increased levels of metadrenalines were detected. This increase was variable and difficult to interpret.

One of the earliest observations on the possible importance of catecholamines in shock syndromes associated with parasitic diseases was made on puppies infected with *Babesia canis* [214]. In one experiment a puppy infected 6 days previously and showing severe shock symptoms, having been unconscious for several hours was given an injection of noradrenaline (32 $\mu$g/ml) into the femoral vein. The description given by these authors is interesting: 'It lay flaccid throughout the operation, with eyes closed and with fast light breathing, rapid heart beat and pale cold extremities and tongue. After 2 ml of the solution had been injected at the rate of 1.0 ml per minute, the eyes opened and the animal stirred and tried to get up. By the time 10 ml had been administered it was fully conscious and able to stand.' These authors believe that the action of noradrenaline is largely due to its vasoconstrictor activity

whereby the smaller vessels are constricted causing the blood to accumulate in the general circulation rather than the peripheral circulation. The effects on cardiac output by noradrenaline were not considered important at the concentrations used. *P. knowlesi* infections of monkeys are similar to *B. canis* of dogs and studies on the portal circulation have been undertaken using angiographic techniques [215–218]. In all the infected monkeys studied the portal vein and its branches were constricted, reducing blood flow and increasing portal pressure. This vasoconstriction was relieved by the ganglion blocking drug, hexamethanium bromide (0.35 mg/kg) or by an adrenergic blocking drug, phenoxybenzamine (1.5 mg/kg). In a further series of experiments the guts of normal monkeys were deliberately manipulated to induce shock. Vasoconstriction was seen in these animals similar to that found in malarious animals and again these changes were prevented by an adrenergic blocking drug. Intestinal circulation of infected monkeys can also be improved by phenoxybenzamine [219].

In both blackwater fever and acute *P. falciparum* malaria, a reduction in kidney cortical blood flow produced renal failure [220, 221]. In human malaria it has recently been shown that vasoconstriction can be relieved by phenoxybenzamine [222] indicating a similar mechanism to the simian disease.

A similar angiographic study has been undertaken in rabbits infected with *T. brucei* [223]. In non-infected rabbits, the central artery of the ear appears uniformly wide, about 1 mm in diameter until it begins to narrow 2–3 cm from its tip. Branches of the central artery occur at intervals along its length which anastomose with the peripheral veins. In the infected animals, the central artery was greatly constricted (0.3–0.8 mm) and the lateral branches were almost or completely closed. Injection of adrenaline (3 $\mu$g/kg) produced a similar picture in the control animals. Hexamethanium (0.2 mg/kg), phenoxybenzamine (1 mg/kg) and pronethanol hydrochloride (0.2 mg/kg) dilated the constricted vessels of the infected animal indicating that adrenergic mechanisms were involved. Also of interest is the fact that atropine or hyoscine hydrobromide (1 mg/kg) also caused the spasm of the artery to relax but the action of these drugs was brief and within 20 min the effects had worn off [223, 224]. This observation supports the conclusion that there is a cholinergic link in the sympathetic fibres to the blood vessels of rabbits' ears [225–230].

Another interesting observation is that liver necrosis in monkeys infected with *P. knowlesi* can be prevented by thoracic sympathectomy before infection [231]. Tissue damage, particularly renal cortical and

hepatic centrilobular necrosis, is a common feature of many shock syndromes including viral, bacterial and protozoal infections and trauma. Local vascular derangements can upset adrenal medullary function [232] and lead to adrenal medullary-cortical-hormone imbalance in human malaria [233]. Recently it has been shown that endotoxin shock has similar effects [234]. Dogs injected intravenously with gram negative endotoxin showed an immediate and precipitous decline in arterial pressure, a simultaneous elevation of portal venous pressure, and a marked decrease in venous return and cardiac output. This author concluded that the progressive development of splanchnic pooling in shock may be due mainly to active constriction of small veins on the basis of increased responsiveness to circulating catecholamines. Changes in adrenal gland histology have not been reported in *B. canis* and *B. argentina* infections, although extensive centrilobular degenerative lesions of the liver and kidney cortical congestion and damage have been seen [214, 235, 236].

## ACETYLCHOLINE AND ACETYLCHOLINESTERASE

A comprehensive summary of the properties of acetylcholine (Ach) falls outside the scope of this review since no studies have been undertaken on its possible significance in parasitic infections. In the body, Ach is rapidly hydrolysed to choline and acetic acid by a group of enzymes, the cholinesterases [237]. Acetylcholinesterase (AchE) has the greatest activity against Ach and is found in plasma and is associated with membranes and cytoplasm of many cells. Studies on AchE in hosts infected with parasites have not been undertaken but in recent years numerous investigations into AchE in helminths have been made.

AchE has been detected both in adult and some 4th stage larval nematodes. It is also found in the secretions of the parasitic stages of some nematodes but very little appears to be secreted by the 3rd stage larva. Most somatic AchE is associated with either the oesophageal region or the excretory gland of worms, although in *Ascaris lumbricoides* [238] low levels of AchE were detected, mainly in association with the nervous system and innervation of muscles. In *Necator americanus*, the hook worm, AchE has also been found in the amphidial glands [239, 240].

High levels of AchE occur in the secretions of the following 4th stage larvae or adult nematode worms: *Cooperia ovina*, *Cooperia pectinata*, *Haemonchus contortus*, *Haemonchus placei*, *Nematospiroides dubius*,

*Nippostrongylus brasiliensis, Oesophagostomum radiatum, Oesophagostomum venulosum, Ostertagia circumcincta, Trichostrongylus axei, T. colubriformis* and *T. refortaeformis* [241–243]. AchE has been described in *N. brasiliensis* and it is suggested that it is immunogenic to its hosts and may be part of the functional antigens [244–247]. A theory has been proposed to explain the presence in large amounts of AchE in helminth infections [152]. It suggests that in *N. brasiliensis* infections, AchE acts as a 'biochemical holdfast' and that its secretion by parasitic stages causes rapid denaturation of Ach, hence preventing the intestinal smooth muscle from contracting and expelling attached worms. No other pharmacological action has been ascribed to the presence of this enzyme in helminth infections, so its role as in some other tissues such as red cell membranes remains in doubt.

## SLOW REACTING SUBSTANCE OF ANAPHYLAXIS (SRS-A)

SRS-A was first described in 1938 [248] during experiments in which guinea-pig lung was perfused with cobra venom. A substance was present in the perfusate which slowly contracted guinea-pig ileum. Later work showed that this substance was released in anaphylactic shock [106, 249, 250]. Its structure is unknown but it is an acidic unsaturated low molecular weight compound containing both hydroxyl and carboxylic acid groups [251, 252].

SRS-A release is an allergen-IgE-mediated mast cell discharge without the presence of either neutrophils or an intact complement system [253]. Thus, the involvement of reaginic antibody in some nematode infections and the concurrent degranulation of mast cells during worm expulsion indicates that SRS-A might also be involved, along with amines. This suggestion has been made to explain a possible mechanism for worm expulsion [177]. In bovines at least [114–116] it has been shown that the main mediators of anaphylaxis are SRS-A and possibly prostaglandins and kinins. Therefore, in infections such as *D. viviparus*, it is likely that SRS-A release occurs. In both *T. evansi*-infected guinea pigs [77] and in chronic *T. brucei* infections [213], the suggestion has been made that SRS-A could be involved. Certainly, as antigen–antibody reactions have been shown to cause SRS-A release [246], the analogy [213] between chronic relapsing *T. brucei* infections which involves antigen–antibody reactions and SRS-A release may indeed be valid.

## THE PROSTAGLANDINS

In the last few years, tremendous advances have been made in the chemistry and pharmacology of prostaglandins [254–257]. Prostaglandins, whose structure is based on prostanoic acid, are fatty acids of 20 carbon atoms. They were widely distributed in a variety of animal tissues [258, 259], show a number of biological properties [260] and have been suggested as therapeutic agents for many medical conditions.

Recent evidence suggests that prostaglandins exert their action via a second agent–cyclic AMP [261]. There are suggestions that prostaglandins may be involved in the pathological processes of cholera, a bacterial disease characterised by a profuse watery diarrhoea. Cholera toxin stimulates the transmucosal secretion of water and electrolytes in dog jejunum [262] and also increases the activity of intestinal adenyl cyclase [263]. These observations led Bennett [264] to propose the hypothesis that, in cholera, the toxin led to increases in the mucosal prostaglandin level which stimulated the intestinal mucosal adenyl cyclase, thus resulting in a raised intracellular cyclic AMP level and increased water and electrolyte secretion. If this theory proves to be correct, this will be the first instance of the involvement of prostaglandins in a disease caused by a micro-organism.

Recently, circumstantial evidence that prostaglandins may be involved in at least *N. brasiliensis* infections has been presented. The intraduodenal injection, on day 6 of infection in rats, of a chloroform extract of ram semen (which contained prostaglandin-like factors) caused the expulsion of the worms from the small intestine. Administration of aspirin and (+)-propoxyphene (inhibitors of prostaglandin) prevented expulsion of the worms [265]. Analysis of these extracts showed that they contained virtually no amines so prostaglandins appeared to be the only pharmacologically active agent involved [266].

In a parallel study, the intraduodenal injection of an inhibitor of prostaglandin isolated from ram semen prevented the expulsion of *N. brasiliensis* from rats between days 10 and 16 of a primary infection [267]. This provided evidence that prostaglandins play an important part in worm expulsion during normal infections. Further evidence for the role of prostaglandins in *N. brasiliensis* expulsion from the rat was obtained by studying the relative activities of synthetic prostaglandins in worm expulsion [193]. The effects of different prostaglandins on worm expulsion were compared by intraduodenal injection on day 6 of the primary infection. Prostaglandins of the A and B classes were less effective than those from the E class. The F class were less effective

again, but still caused a modest reduction in worm numbers. However, although prostaglandins were found to be highly effective in the expulsion of worms on day 6 (when histamine and 5-HT were ineffective), these data do not preclude the involvement of amines during the normal expulsion process on days 10–16.

## MISCELLANEOUS FACTORS

Although there is no direct evidence, it is possible that adenosine or related compounds may be released in some parasitic infections. This suggestion is based on an early observation that these substances were present in the venous blood returning from the ischaemic hind limb of a rabbit, produced by trying the common iliac artery [268].

In a study of the levels of adenosine phosphatases in erythrocytes of *P. knowlesi*-infected monkeys, increased amounts were found [269]. It was considered that this increase was probably associated with the growth and development of the parasite to the large trophozoite stage. Thus the changes in adenosine phosphatases seen in the erythrocyte membrane probably reflect metabolic changes by the parasite rather than release from the host.

Increases in ATPase have also been reported in erythrocyte membranes of young calves infected with *Anaplasma marginale* [270]. It was considered that this change was entirely related to membrane function rather than having pharmacological implications.

Sera taken from rhesus monkeys infected with *Plasmodium inui* or *P. coatneyi* caused an increase in vascular permeability when injected intradermally into rabbits [271]. This response was inhibited by promethazine (10 mg/kg). The exact nature of this permeability factor is unknown; the authors considered that it could well be related to the pharmacologically active peptides, even though it was inhibited by antihistamines.

During studies on the release of pharmacologically active substances in various protozoal infections, it has been found that sera taken from these animals potentiates the effects of peptides and amines on isolated tissues. For example, sera from mice infected with *B. rodhaini* potentiate histamine on the guinea-pig ileum [51] and sera from animals infected with *T. brucei* potentiate histamine, 5-HT, Ach and bradykinin [272]. Likewise, sera of guinea-pigs infected with *T. evansi* potentiate Ach and histamine [77]. No mechanisms have been postulated to explain these observations.

When diffusion chambers containing *T. congolense* are implanted intraperitoneally into rats, a severe local inflammatory reaction results [273]. This reaction only occurs when living parasites are present and is not present when *T. lewisi* is used. The inflammatory reaction is not enhanced by using previously immunised rats. Thus, it appears that biologically active factor(s) are released from dying *T. congolense* which are capable of initiating an inflammatory reaction.

There are other pharmacologically active substances which have not been looked for in parasitic infections, in particular, angiotensin and substance P. As kidney damage has been shown to be an important factor in some blood-borne protozoan infections [213, 218, 235], it is possible that angiotensin, at least, would be released and contribute to the pathological processes.

## RELEASE OF PHARMACOLOGICALLY ACTIVE SUBSTANCES AFTER CHEMOTHERAPY

As early as 1929, an observation was made which could well have major significance in the treatment of parasitic diseases but to date has not really been followed up [274]. Stephan and Esquibel found that when they treated cattle infected with piroplasms with a non-toxic dose of euflavine, the parasites were cleared from the blood, but after 24 h the animals died. This state resembles that found in laboratory animals infected with piroplasms or trypanosomes and treated with non-toxic doses of drugs [275]. Similar findings have been reported in a number of bacterial diseases including typhoid fever treated with chloramphenicol [276, 277] and human brucellosis treated with chlortetracycline [278]. The situation is similar to that in the Herxheimer reaction of syphilis [279, 280] which has also been seen in human trypanosomiasis [281, 282]. It seems likely that the release of pharmacologically active substances, possibly as the result of liberation of parasite antigen or an immune reaction, are important.

In none of these studies was measurement of pharmacologically active substances made. However, Desowitz has recently being studying dogs infected with *D. immitis*. On treatment with diethylcarbamazine, there was a rapid thrombocytopaenia and increase in plasma 5-HT levels. He concluded that as the result of an immune reaction 5-HT was being liberated from platelets [283]. Clearly this field requires more extensive study.

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# 3 The Medicinal Chemistry of 1,2,3-Triazines

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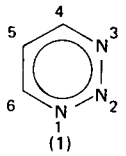
## INTRODUCTION

It is unusual for a review to be written for this Series on a class of compounds which have yet to find a commercial medicinal application. Accordingly, the reader may well conclude that this article is premature and should be treated with caution. Nevertheless, there are features of the medicinal chemistry of 1,2,3-triazines which give confidence that useful biological activity will be discovered in the series, and the attention of the reader will be focussed on these features.

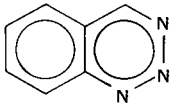


(1)

The parent heterocycle 1,2,3-triazine (1), also known as *v*-triazine, has never been prepared and there are (literally) only a handful of reports describing the synthesis and structure of some simple 4,5,6-trisubstituted derivatives [1–5]. Photolytic [2] and thermolytic [6] degradation of the NNN linkage are the only notable properties of these compounds.

However, when the 1,2,3-triazine nucleus (1) is annelated at the 5,6-(or 4,5-) positions with an *aromatic* carbocyclic or heterocyclic moiety, the compounds have an added dimension to their reactivity–they behave as masked diazonium compounds [7, 8]. This property confers on the products the potential for versatile interactions with biologically significant macromolecules; this potential has been little examined and certainly not yet realised.

Accordingly, this review will concentrate on the medicinal chemistry of 1,2,3-benzotriazines (2), benzotriazines (3) fused at the *c* bond to an azino- or azolo-ring (*x*), and the important series of hetero-fused 1,2,3-triazines (4) where *y* signifies a hetero-aromatic 5- or 6-membered ring. Other rarer tri- and polycyclic triazines illustrated by types (5) and (6) will also be mentioned.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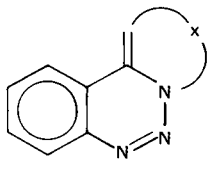
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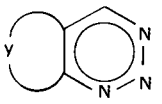
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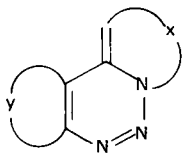
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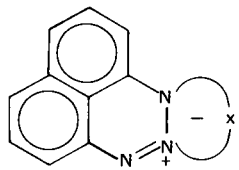
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Because the chemistry of all these fused triazines is dominated by their diazonium character, it is appropriate at first to briefly summarise some of the chemical and biological properties of diazonium compounds.

# CHEMISTRY OF ARYLDIAZONIUM COMPOUNDS

In the field of organic chemistry, there are few reactions which can be relied upon with more certainty than the formation of a diazonium compound by the action of nitrous acid on a primary arylamine [9–14]. The study of diazonium species is almost as old as organic chemistry, and their structure can be represented by the principal contributing mesomers (7a and b).

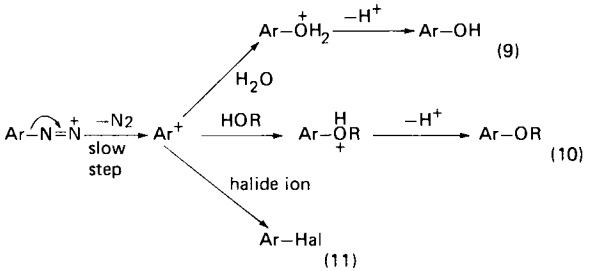
(7a) (7b)

Aromatic diazonium salts are generally of low stability unless the aryl group is substituted in *ortho* or *para* positions with substituents with lone electon pairs (e.g. ÖH, ÖAlk, S̈H, S̈Alk, N̈H$_2$ or N̈Alk$_2$). In these cases, resonance interaction (+ M effect) increases double bond character at the bond linking the diazo group to the aryl moiety and renders it less liable to fission (e.g. 8a↔8b).

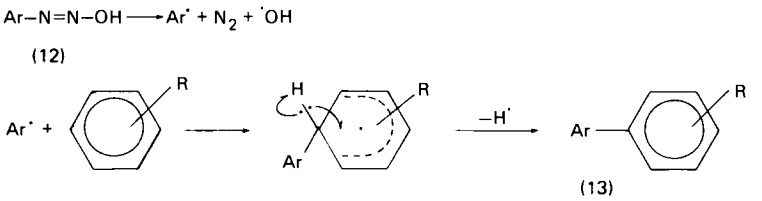
(8a) (8b)

Likewise, *p*-tolyldiazonium chloride decomposes more slowly than benzenediazonium chloride, presumably because hyperconjugation strengthens the C–N bond in the tolyl compound [15].

The reactions of diazonium compounds can be conveniently classified into two types: those in which the N atoms of the diazo group are lost in the product, and those in which the N atoms are retained. In protic solvents, particularly in acidic aqueous media, decomposition of diazonium compounds with loss of nitrogen proceeds via an $S_N1$ reaction. The rate-determining step is heterolytic cleavage of the C–N bond leading to an aryl cation ($Ar^+$) which rapidly reacts with available nucleophiles; typical products include phenols (9), ethers (10) and aryl halides (11). Electron-donating groups in the aryl fragment accelerate reactions, and electron-withdrawing substituents retard reaction rates (with exceptions outlined above) [15–17].

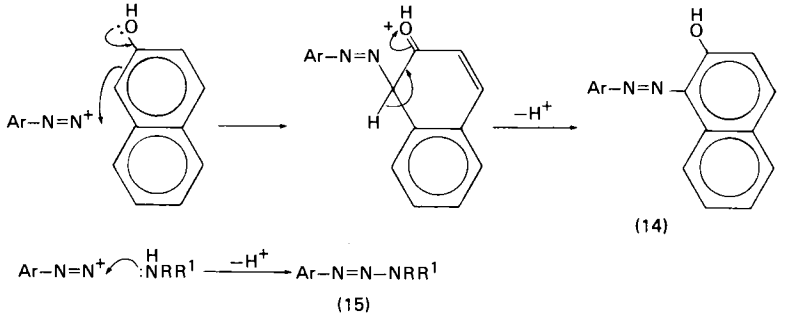



In contrast, decomposition in neutral, basic or non-polar media proceeds homolytically via the intermediacy of aryldiazohydroxides (12). The reactivity of neutral aryl radicals (Ar$^{\cdot}$) formed in this process can be exploited in the Gomberg synthesis of biaryls (13) [18, 19] and other reactions with a range of substrates [20–23].



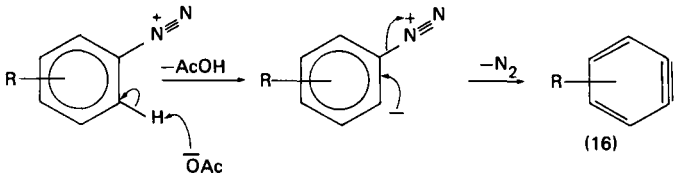

Decomposition of *p*-(*NN*-dimethylamino)benzenediazonium chloride under the influence of ultraviolet light also proceeds by a radical process [24]. It is possible that conditions for homolytic fission of the diazo group may occur in the non-polar microenvironments of hydrophobic regions on biological macromolecules (e.g. enzymes or antibodies).

Examples of reactions involving retention of nitrogen are the familiar coupling between diazonium salts and the activated aromatic nucleus of 2-naphthol to form an azonaphthol dye (14), and the reaction with amines



to afford substituted triazenes (15) where R and R' can be H, alkyl, aralkyl, aryl, heteroalicyclic or heteroaromatic groups or combinations of the same [25–27]. Analogous coupling reactions between diazonium compounds and alcohols and phenols to yield diazoethers [28, 29], with thiols to afford diazothioethers, as well as reactions with numerous other nucleophiles have been reviewed [10, 11].

Finally, it has been recently established [30] that benzenediazonium acetate decomposes by an alternative process to yield benzyne, and a method for a 'one-pot' conversion of arylamines to reactive aryne intermediates (16) has been devised [31, 32]. It is unlikely that this decomposition has any relevance in terms of the biological activity of diazonium compounds as the reaction is inhibited by water.

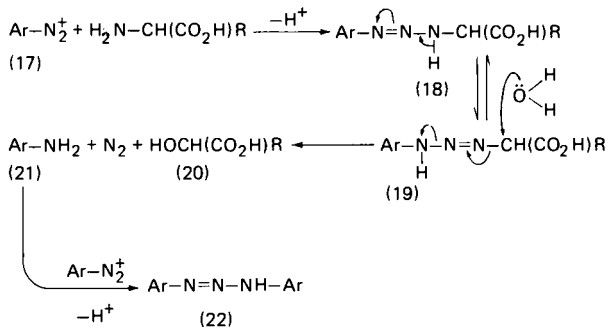



## REACTIONS OF DIAZONIUM COMPOUNDS WITH BIOLOGICALLY IMPORTANT MOLECULES

### AMINO ACIDS AND PROTEINS

Because of their promiscuous chemical nature, it is not surprising that a study of the interactions of diazonium compounds with biological macromolecules has attracted widespread interest. In his pioneering work in chemical immunology, Landsteiner introduced small haptenic groups into proteins by means of diazonium coupling reactions and studied the modified antigenicity of the coupled products [33]. Early work on amino acid–diazonium interactions has been reviewed [34], and recent efforts have been directed towards elucidating the structures of the products.

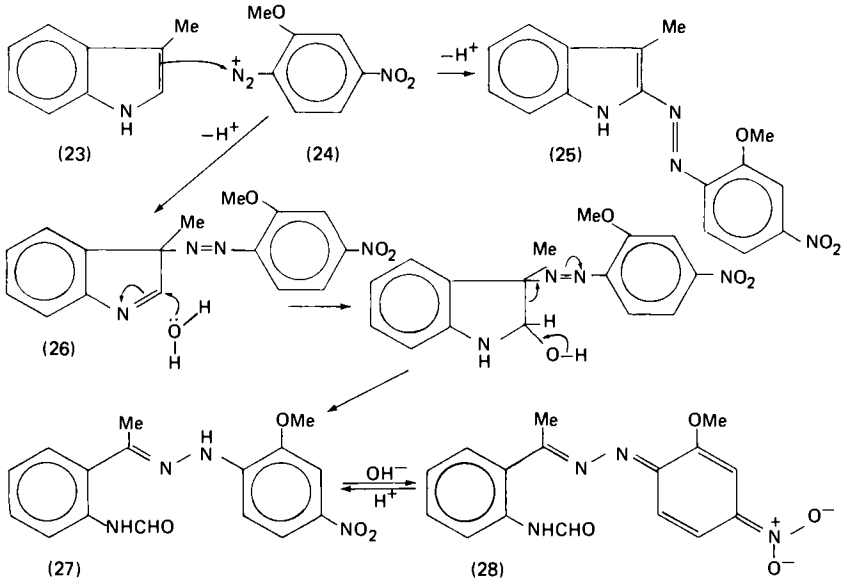
A distinction must be made between reactions of diazonium salts at the α-amino group of free α-amino acids and reactions at the nucleophilic side chains of amino acids in peptides and proteins. Deamination of the α-amino acids is usually observed in the former cases as in the reactions of diazotised 4-chloro-*o*-toluidine with glycine, DL-alanine, DL-isoleucine and L-glutamic acid [35].




The general reaction is described in formulae (17)–(22); coupling between the diazonium salt (17) and the $\alpha$-amino acid first yields the triazene (18); hydrolytic decomposition of the unstable tautomer (19) leads to the formation of an $\alpha$-hydroxy acid (20) and the regeneration of arylamine (21). This in turn couples with the excess of the original diazonium salt affording a diazoamino compound, the diaryltriazene (22). The net result is conversion of an arylamine to a diaryltriazene with concomitant deamination of an $\alpha$-amino acid, and the process has been described as 'diazo migration' by Zollinger [36]. The stereochemical features of the deamination step are puzzling. If hydrolytic breakdown occurs by an $S_N2$ reaction as depicted above, an $\alpha$-hydroxy acid with opposite configuration to the starting $\alpha$-amino acid would result. However, recent work on the decomposition of optically active triazenes has shown that interceptible carbenium ions are formed, and that *retention* of configuration in the substitution products is observed [37, 38]. Presumably, as with other nucleophilic substitutions, the mechanism of the reaction will be modified by different substituents on the carbon bearing the leaving group [39].

When the amino acids have their $\alpha$-amino groups blocked, or are components of peptides and proteins, the vulnerable targets for aryldiazonium salts are the activated aromatic rings of tyrosine and histidine residues and the $\epsilon$-amino group of lysines [40–46]. Evidence has been adduced for the formation of mono- and bis-azo derivatives in each case and the quantitative aspects of the coupling are critically dependent on pH. The chemistry of penta-azadienes said to be formed in couplings with lysine residues is an area which has been little studied and which merits further examination [47]. Reviews on the use of diazonium salts as staining reagents in cytochemistry [48] and on the applications of ligand coupling via the azo linkage in affinity chromatography [49] stress the importance of reactions with tyrosine and histidine.

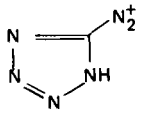
Although nitrosation of tryptophan derivatives is known to involve the indolic NH group [50, 51], coupling of tryptophan residues in proteins with aryldiazonium salts is imperfectly understood [41, 52, 53]. Indole itself undergoes coupling in the 3-position [54] whereas skatole (23) reacts with diazotised 2-methoxy-4-nitroaniline (24) to form the 2-azo derivative (25) [55] and the hydrazone (27) [56]: the proportions of the two products depend on the pH of the medium, an acid pH favouring azoindole (25) and a neutral pH leading to a preponderance of hydrazone (27). It seems likely that the hydrazone is derived from the arylazoindolenine (26) which would be susceptible to hydrolytic ring opening [56]. The nitrohydrazone (27) forms a magenta-coloured nitronate anion (28) in alkali, and if a similar coupling and ring opening reaction occurred with tryptophan derivatives in proteins, a quantitative colorimetric determination of tryptophan residues could be developed. Tryptophan in proteins may well be more reactive than hithertofore thought [57, 58].




In recent years, attention has been focussed on exploiting the activity of aryldiazonium compounds to elicit information about the structure of physiologically active proteins, (and) the topography of the active sites of enzymes and the combining regions of antibodies. In many of these studies specific amino acids have been identified and shown to have an

important role in either promoting substrate–macromolecule association or to be involved in the molecular events in catalytic activity. Some labelled macromolecules include insulin [59], human erythrocyte membrane [60], the sulphate binding protein of *Salmonella typhimurium* [61], the enzymes trypsin [62], chymotrypsinogen A and $\alpha$-chymotrypsin [63], carboxypeptidase A [64–67], rabbit liver fructose diphosphatase [68] and myosin A ATPase [69, 70]. In most of these cases, the labelled amino acids have been tentatively identified as tyrosine, histidine and lysine.

The heterocyclic diazonium salt, diazonium-1*H*-tetrazole (29) (DHT), is a useful histidine-modifying reagent which can discriminate between exposed and buried histidine residues; it is therefore a useful probe for the surface areas of proteins [71].



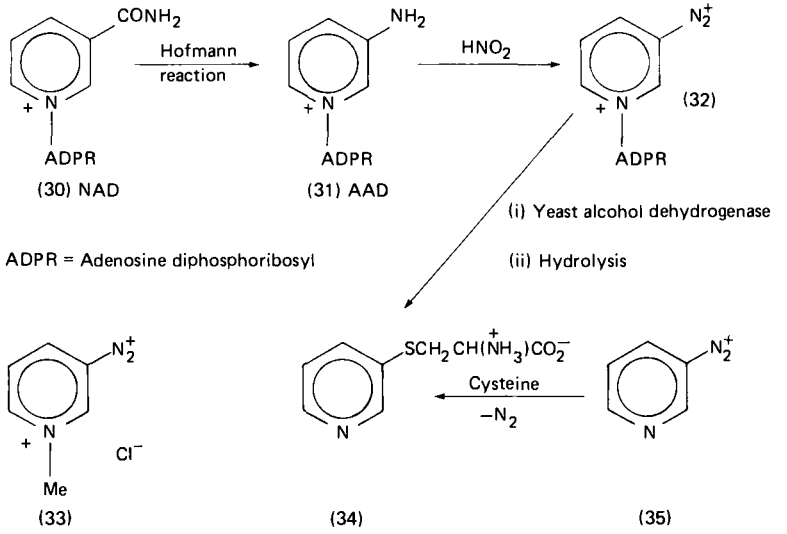
(29)

The chemical basis of the procedure involves the spectrophotometric determination of histidine bisazo-1*H*-tetrazole (at 480 nm) formed when a protein solution is coupled with DHT. Tyrosine residues are less reactive and buried histidine units inert [71]. The four histidine residues of ribonuclease couple in discrete steps whereas, as expected, no differential reactivities are observed for the denatured enzyme [72]. The environments of histidine in bacitracin, insulin, lysosyme, bovine serum albumin, trypsinogen and trypsin [71], cytochrome *c* [73], chymotrypsin [74], carboxypeptidase A [75] and glucose dehydrogenase [76] have been probed and the method reviewed [77, 78]. Recent work casts doubt on the specificity of the coupling however. DHT has been shown to react with papain and succinylpapain to modify histidine, tyrosine, tryptophan and lysine residues [79].

Certain diazonium compounds come into the category of what Baker has defined as 'active-site-directed irreversible enzyme inhibitors' [80]. In this approach the diazonium compound is designed with structural components which are complementary to binding regions on the surface of the target molecule. In principle, prior formation of an inhibitor–macromolecule complex will allow greater specificity to be achieved in subsequent covalent reactions than in the purely bimolecular processes involved in the reactions of non-directed aryl- and hetaryldiazonium compounds. Two examples serve to illustrate the point.

NAD (30) can be efficiently converted to 3-aminopyridine adenine

dinucleotide (AAD) (31) by a Hofmann hypobromite reaction [81]. Diazotised AAD (32) is a site-specific inactivator of yeast alcohol dehydrogenase whereas the model diazopyridinium chloride (33) has only 1/10th of its effectiveness. In a follow-up study [82] diazotised AAD (32) was shown to modify four sulphydryl groups per molecule of enzyme as deduced by titration with 5,5′-dithiobis(2-nitrobenzoic acid). With both diazonium compounds, NAD protects the enzyme against inactivation: by implication, the sulphydryl groups of yeast alcohol dehydrogenase must be located near the pyridinium ring region of the coenzyme binding site.




Surprisingly, *S*-(3-pyridyl)cysteine (34) was isolated by hydrolysis of the modified enzyme and shown to be identical with the product formed from diazotised 3-aminopyridine (35) and cysteine. Useful further work in this area could be directed towards investigating the nature of the reactive species involved and the timing of the loss of nitrogen. Diazotised AAD (32) and the model pyridinium salt (33) also react with homocysteine and glutathione under mild conditions [81, 82]. Cysteine sulphydryl groups in proteins may be more reactive than generally considered [83].

Another study [57] has yielded important information about the amino acid residues at or near the active site of staphylococcal nuclease and illustrates the selectivity that may be achieved in the active-site-directed approach. Of three diazotised deoxythymidine derivatives (see *Figure* 3.1) used in this work, Reagent *a* reacts entirely with tyrosine-115; the

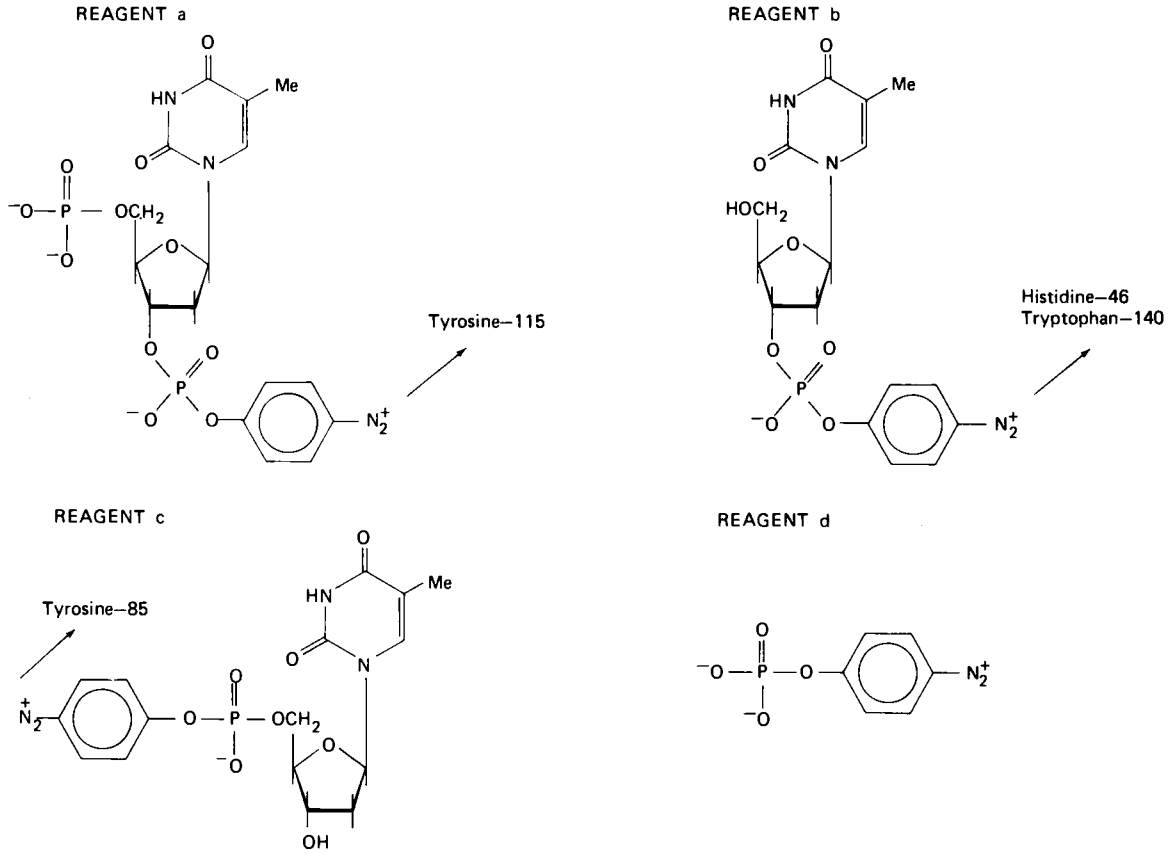



*Figure 3.1. Reagents used in the labelling of staphylococcal nuclease: diazotised deoxythymidine 3'-*p*-aminophenyl phosphate 5'-phosphate (a); diazotised deoxythymidine 3'-*p*-aminophenyl phosphate (b); diazotised deoxythymidine 5'-*p*-aminophenyl phosphate (c); and diazotised* p*-aminophenyl phosphate (d)*

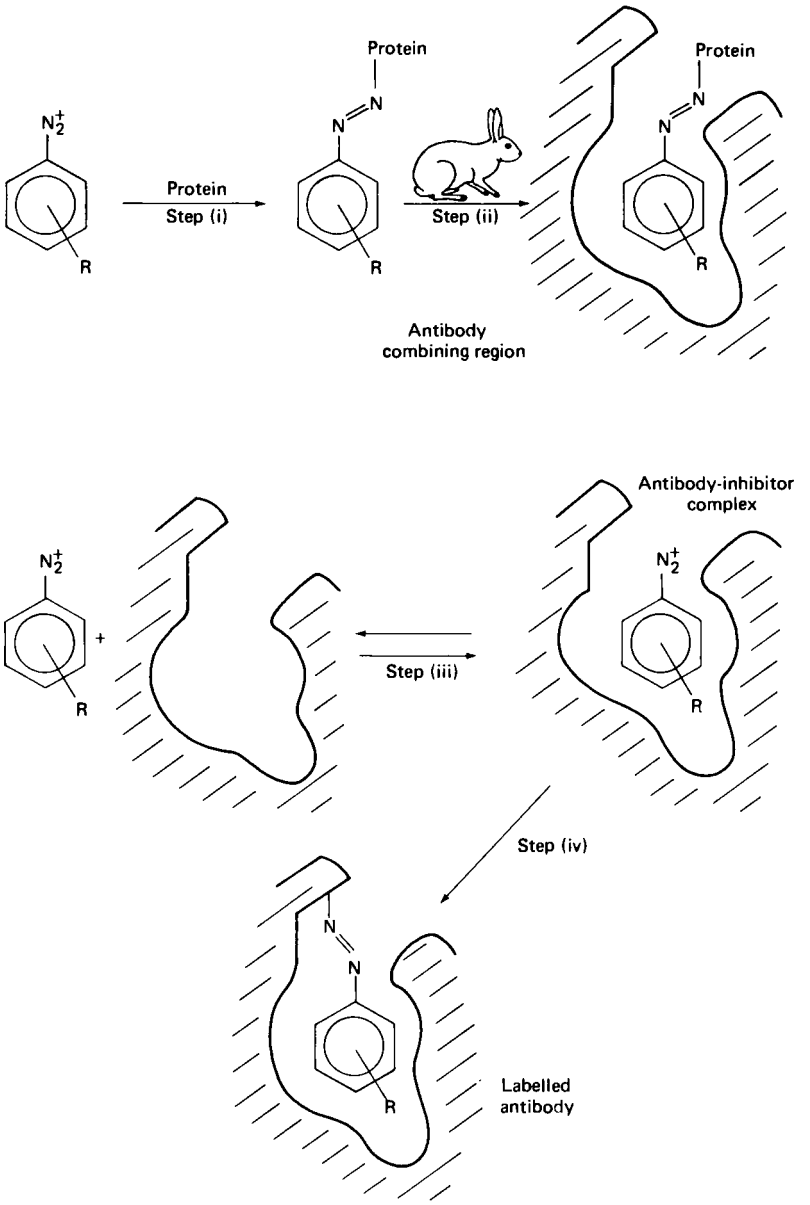



*Figure 3.2. Affinity-labelling of antibodies with aryldiazonium salts*

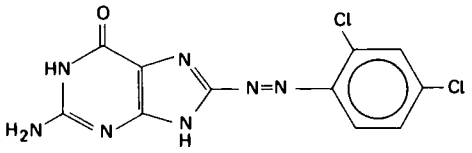
closely related Reagent *b* reacts with both histidine-46 (55%) and tryptophan-140 (45%); Reagent *c* on the other hand labels only tyrosine-85. The model diazonium compound (Reagent *d*) is, as expected, indiscriminate it its labelling patterns.

An analogous approach ('affinity-labelling') was devised by Wofsy, Metzger and Singer [84] to label the combining regions of antibodies which, as non-catalysts, lack uniquely reactive groups. Affinity-labelling can be summarised in four steps (*Figure 3.2*). In step (i), the appropriate aryldiazonium moiety is coupled with a protein to form an azo-compound; this derivative acts as an antigen when injected into an animal, e.g. rabbit, in step (ii). Antibodies are elaborated which have combining regions specific for the recognition of the arylazo grouping. When a suitable antibody titre has been achieved the animals are sacrificed; the antibody is isolated, purified, and then treated in step (iii) with the original diazonium compound. The diazonium group, constrained within the antibody combining region in the inhibitor–antibody complex, should then react covalent in step (iv) with whatever reactive amino acid side-chain is in the vicinity. This residue can be identified by hydrolytic degradation of the covalently-labelled complex. In general, tyrosine and histidine units are marked [85, 86]. There is continuing interest in the method [87–92] and an important recent development has been the design of 'photo-affinity reagents'. These reagents (generally aliphatic diazo compounds, or aromatic azides) only develop their covalent labelling capability on photolysis. The reactive intermediates (carbenes and nitrenes respectively) have a broader spectrum of chemical activity than the aryldiazonium compounds of the traditional method [93].

## NUCLEIC ACID BASES

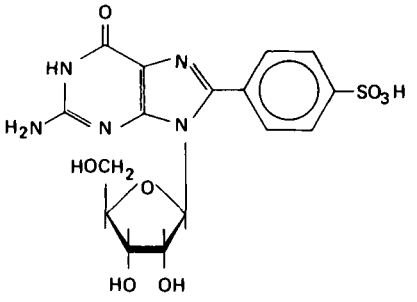
In contrast to the considerable knowledge on diazonium–protein reactions, there is a paucity of information on the corresponding reactions with nucleic acid bases. This is surprising since profound effects on cell function could be elicited by such interactions if they occurred in vivo.

Fischer [94] in 1903 coupled diazonium compounds with the purines theophylline, xanthine and guanine; the product from guanine and 2,4-dichlorobenzenediazonium chloride was the 8-azoguanine (36). Diazotised sulphanilic acid couples with guanylic, adenylic and cytidylic acids at pH 10–11 to afford pigments ($\lambda_{max}$ 370–440 nm) whereas uridylic and thymidylic acids do not react [95, 96]. Because pigment formation



(36)

was inhibited by formaldehyde (which blocks amino groups), it was concluded that the products were diazoamino compounds (triazenes). The higher ordered structure of intact nucleic acids does not protect the bases from attack by diazonium compounds: the amino groups of guanine and adenine residues (not cytosine) in RNA and DNA couple with diazotised sulphanilic acid [97] during which the DNA double helix is disrupted. Diazotised sulphanilic acid is mutagenic towards phage $\phi$X 174 at pH 8 and 2°C [97]. Two surprising by-products were identified in the coupling of the same diazonium salt with guanosine at pH 9, in addition to the expected triazenes formed by reaction at the 2-amino group [98]. It is tempting to speculate that these by-products (37) and (38) might arise by homolytic arylation at C-8 in the manner of the Gomberg biaryl synthesis [18, 19]: certainly, the reaction conditions would favour a radical process.



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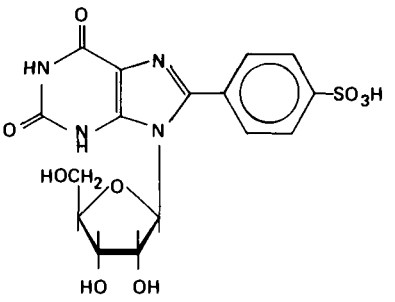


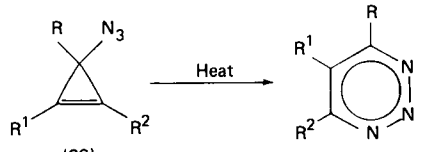
(38)

Beer and Moudrianakis [99] have attempted to exploit diazonium coupling reactions to determine base sequences in DNA. At pH 9, guanylic acid residues react sixty times faster than any of the other common nucleotides with diazotised 2-aminobenzene-1,4-disulphonic acid [100].

Early work on coupling reactions between diazonium salts and purines and pyrimidines has been reviewed [100].

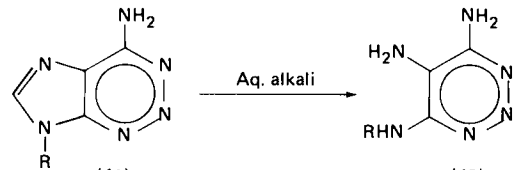
## THE CHEMISTRY OF 1,2,3-TRIAZINES

There are only two authenticated routes to monocyclic 1,2,3-triazines. The more versatile route involves the thermal rearrangement of trisubstituted cyclopropenyl azides (39) which readily afford 4,5,6-trialkyl- or 4,5,6-triaryl-1,2,3-triazines (40) [1–3]. The structure of the tri-*p*-methoxyphenyl analogue (40; R = R$^1$ = R$^2$ = *p*-methoxyphenyl) has been corroborated by X-ray crystallography [4].




(39) (40)

The intriguing decomposition of 9-cyclopentyl-2-aza-adenine (41) in aqueous alkali to the substituted triamino-1,2,3-triazine (42) is the only recorded example of a synthetic method which could presumably be exploited with other 9-substituted 2-aza-adenines [5] (see later).



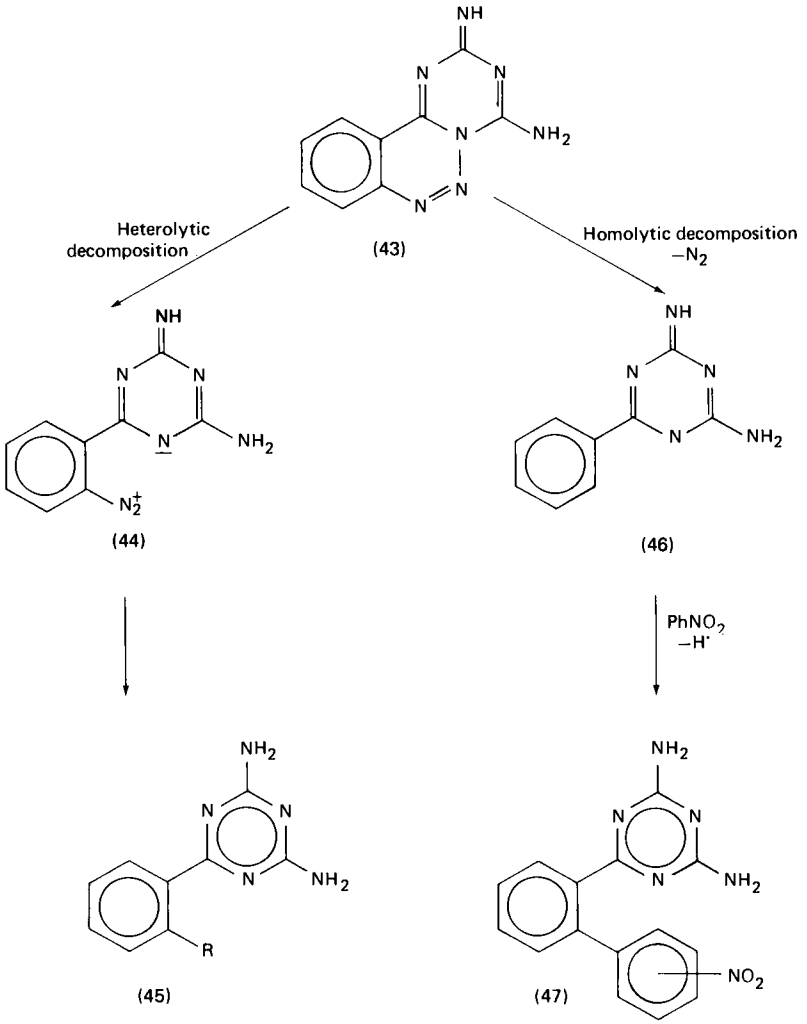

(41) (42)

R = Cyclopentyl

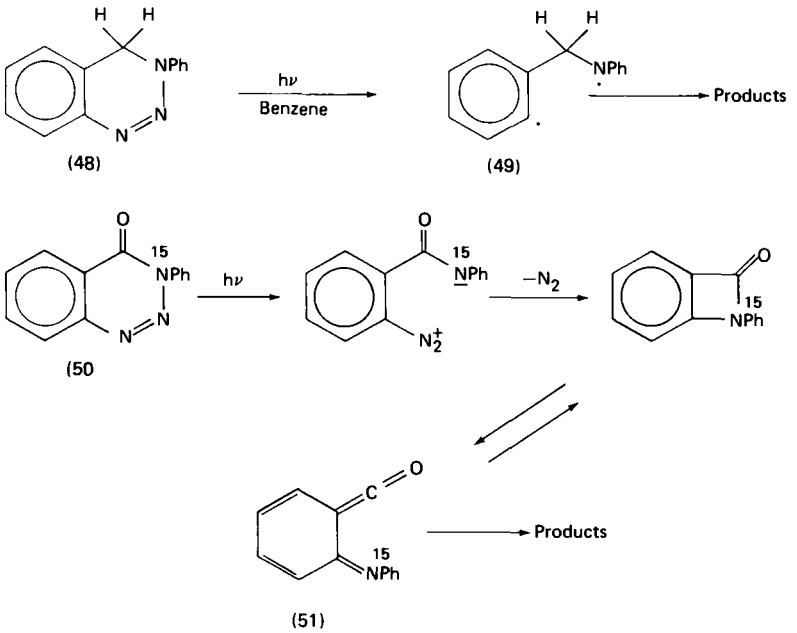
Earlier reviews [7, 8] on annelated 1,2,3-triazines have emphasised their 'masked' diazonium character, and recent work has confirmed that decomposition of these types is dominated by heterolytic or homolytic cleavage of the triazine ring. A complete review of the chemistry of benzo- and hetero-fused 1,2,3-triazines is outside the scope of this work: instead, a few specific cases have been chosen which illustrate the parallel chemistry of fused 1,2,3-triazines and aryldiazonium compounds.

For example, decomposition of 4-amino-2(2*H*)-imino-*s*-triazino-[1,2-*c*][1,2,3]benzotriazine (43) in ethanol alone or in ethanol containing 2-naphthol yields 2,4-diamino-6-phenyl-*s*-triazine (45a) or the corresponding naphtholazo-derivative (45b) respectively [101]; in piperidine, the ring-opened triazene (45c) is formed [102]. These reactions, involving loss or retention of the diazo group are heterolytic in nature and implicate the

diazo species (44) as reactive intermediate. In contrast, thermolysis of benzotriazine (43) in nitrobenzene is undoubtedly homolytic in character [101] since the products are isomeric disubstituted biphenyls (47). An intermediate diradical species (46) is probably involved (cf. the Gomberg reaction [18, 19]).



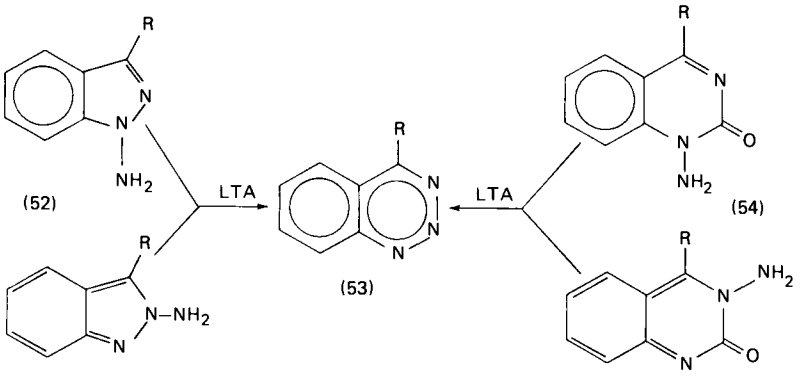
Photolysis of 3,4-dihydro-3-phenyl-1,2,3-benzotriazine (48) in moist benzene [103] affords a mixture of products considered as arising from the diradical species (49), whereas photolysis of 3-phenyl-1,2,3-benzotriazin-4(3*H*)-one (50) gives the keten-imine (51) which can be trapped by nucleophiles and dienophiles; use of $^{15}N$-labelled triazinone confirms that nitrogen is extruded from the 1- and 2-positions by a heterolytic mechanism [104]. The corresponding 3-alkyl- and 3-aralkylbenzotriazinones appear to be photostable [105, 106].




A spate of recent publications on the photolytic [107–112], thermolytic [106, 113–119] and electron-impact promoted [113, 120, 121] decompositions of fused 1,2,3-triazines attests to the continuing interest in the mechanistic aspects of the subject.

Decomposition of 1,2,3-benzotriazines by acids [101, 105, 122–125], reducing agents [105, 107, 125–127], and assorted nucleophiles [102, 107, 116, 122, 128–130] generally proceeds under mild conditions in a manner which closely mimics similar reactions of diazonium compounds: model reactions with nucleophiles have particular significance in view of the availability of reactive nucleophilic sites on biomacromolecules (e.g. enzymes and nucleic acids).

Recent synthetic developments in the 1,2,3-triazine field have, in general, involved adaptation of established routes [7, 8] with the notable exception of the work of Rees and his colleagues who have explored several ingenious pathways to 1,2,3-benzotriazine itself, its 4-substituted derivatives and some heteroaromatic analogues [131]. For example, oxidation of isomeric *N*-aminoindazoles (52) or *N*-aminoquinazolin-2-ones (54) with lead tetra-acetate in methylene chloride affords 4-substituted 1,2,3-benzotriazines (53) in yields which are determined by the nature of substituent R.



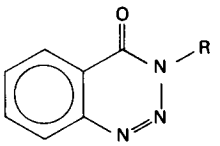

R = H, Me, OMe, Ph, $C_6H_4OMe$–*p*

These advances offer both an entry into novel heterocyclic systems [132, 133] and the prospect of extending the range of 1,2,3-triazines available for biological testing.

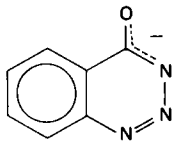
## BIOLOGICAL ACTIVITY OF 1,2,3-TRIAZINES

### 1,2,3-BENZOTRIAZIN-4(3*H*)-ONES

3-Substituted-1,2,3-benzotriazin-4(3*H*)-ones (55) are the most widely studied of all 1,2,3-triazines probably because of their ease of synthesis. Typically, they may be prepared by diazotisation of substituted anthranilamides [7, 8] or by cyclisation of *o*-triazenobenzoate esters [7, 8, 105, 130, 134–136]. Group R can be alkyl, aryl, aralkyl, acyl, aroyl, alkoxycarbonyl, hydroxy, amino or a range of more elaborate substituents.



(55)



(56)

Less reliable as a route to 3-alkylbenzotriazinones is the alkylation of the sodium salt of 1,2,3-benzotriazin-4(3*H*)-one (i.e. the ambident anion (56)) since the required *N*(3) substituted products may be contaminated with other isomers [137, 138]; arylation gives exclusively the *N*(2)-arylated derivatives [139] (see later).

Published information on the biological properties of benzotriazinones is mainly confined to the patents literature: often it is not explicitly stated which compounds have the claimed activity, or whether the activity refers to work in human subjects or experimental animals. These results are presented in *Tables 3.1–3.3.* Not all the compounds mentioned in the patents are listed. In many cases, examples are given which illustrate the structural types with purported activity.

Table 3.1. BIOLOGICAL PROPERTIES OF UNSUBSTITUTED AND 3-ALKYL-1,2,3-BENZOTRIAZIN-4(3*H*)-ONES

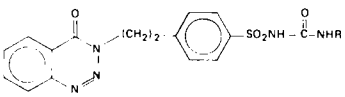
| *Compounds* | *Summary of biological properties* | *References* |
|---|---|---|
| (55) | | |
| R = H | Fertility control in rats | [140] |
| | Sedative activity | [141] |
| R = H, Me, benzyl, phenethyl, (2,4-diaminopyrimidin-5-yl)-methyl | Inactive as anti-tumour agents against lymphoid leukaemia (L 1210) | [142] |
| R = Me, Et, Pr, Pr$^{i}$, 3-methoxypropyl, Bu, Bu$^{t}$, hexyl, benzyl, phenethyl | Antipyretic, analgesic, sedative and anti-inflammatory activity | [143] |
| R = 1-Adamantyl | Potential virostatic agent | [144, 145] |
|  (57) | | |
| R = Cyclohexyl, 4-methylcyclohexyl, Bu | Hypoglycaemic activity | [146] |

Table 3.1. (*cont.*)

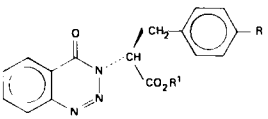
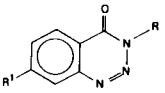
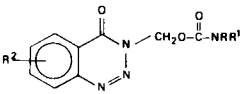
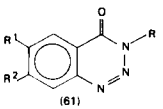
| *Compounds* | *Summary of biological properties* | *References* |
|---|---|---|
|  (58) | | |
| R = H, OH<br>$R^1$ = H, Et | Potential substrates and inhibitors of α-chymotrypsin | [51, 105, 147] |
|  (59) | | |
| R = H, $(CH_2)_2NMe_2$, $(CH_2)_3NMe_2$<br>$R^1$ = Cl | No sedative activity | [141] |
|  (60) | | |
| R = H, Me, Et, Pr, $Pr^i$, Bu, cyclohexyl, $CH_2OH$, Ph<br>$R^1$ = H, Me, Ph<br>$R^2$ = H, Me, Et, Pr, $Bu^s$, Br, I, $NO_2$, OMe in 5,6,7, or 8-positions, 6,8-di- or 6,7,8-tri-substituted derivatives | Tranquilising, muscle relaxant, anticonvulsant, herbicidal, insecticidal and bacteriocidal activity | [148] |
|  (61) | | |
| R = H<br>$R^1$ = H, Cl, OAlk<br>$R^2$ = H, OAlk<br>$R^1R^2$ = Methylenedioxy | Anti-inflammatory activity | [149] |
| R = H, Me, Et, benzyl, $(CH_2)_2NMe_2$, $NH_2$, N = CMePh<br>$R^1$ = $SO_2NH_2$<br>$R^2$ = Cl | Diuretic activity | [150, 151] |

Table 3.1. (*cont.*)

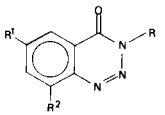
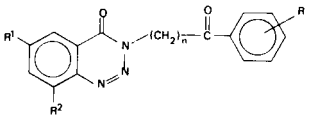
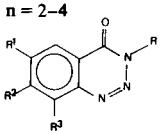
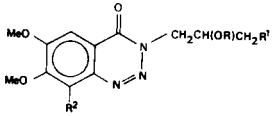
| *Compounds* | *Summary of biological properties* | *References* |
|---|---|---|
|  (62) | | |
| R = H, Me, Et, Pr, Pr$^i$, $CH_2OH$, benzyl, phenethyl, morpholinylmethyl, morpholinylethyl<br>$R^1R^2$ = H, Cl, Br | Sedative activity | [152] |
|  (63) | | |
| R = Lower alkyl, Cl, Br, I, OMe, $CF_3$, cyclic or *non*-cyclic *t*-amino group<br>$R^1R^2$ = H, halogen<br>n = 2–4 | Anticonvulsant, antidepressant, analgesic and psychosedative activity | [153] |
|  (64) | | |
| R = H, Me, Et, Pr$^i$, $CH_2OH$, $(CH_2)_2NMe_2$ morpholinyl, 3-morpholinylpropyl, 2-(hexamethyleneimino)ethyl<br>$R^1R^2$ = Di-OMe; $R^3$ = H, Me<br>$R^1R^2$ = Methylenedioxy; $R^3$ = H, Me<br>$R^1R^2R^3$ = Tri-OMe | Anti-inflammatory activity | [154, 155] |
| R = $(CH_2)_2CO_2H$, $(CH_2)_2CO_2Et$, $CHMeCO_2H$, $CHMeCO_2Et$<br>$R^1$ = H, Cl, OMe, $NO_2$<br>$R^2$ = H, OMe<br>$R^3$ = H, OMe | Anti-inflammatory activity | [156] |
|  (65) | | |
| R = H, 3′,4′,5′-trimethoxybenzoyl<br>$R^1$ = Range of cyclic and non-cyclic t-amino groups<br>$R^2$ = H, OMe | Coronary dilating activity | [157–161] |

Table 3.2. BIOLOGICAL PROPERTIES OF 3-ARYL- AND 3-HETARYL-1,2,3-BENZOTRIAZIN-4(3*H*)-ONES

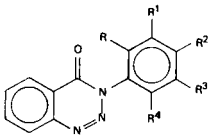
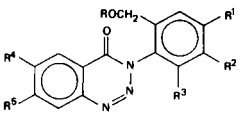
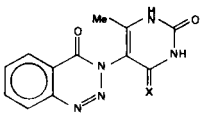
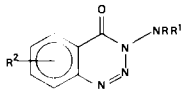
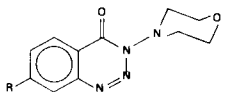
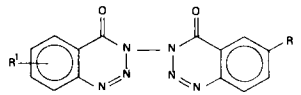
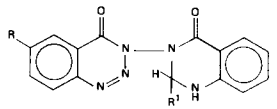
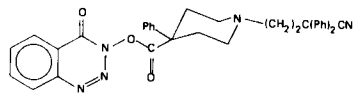
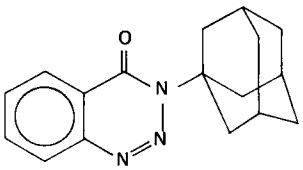
| *Compounds* | *Summary of biological properties* | *References* |
|---|---|---|
|  (66) | | |
| R = F<br>$R^1$–$R^4$ = H | Appetite-suppressant, anticonvulsant and hypoglycaemic activity | [162] |
| R = Me<br>$R^2$ = Cl, OMe, $NEt_2$<br>$R^1R^3R^4$ = H | Sedative, hypnotic and cramp-inhibiting activity | [163] |
| R = H, Cl, Me, $NO_2$<br>$R^1$–$R^4$ = H | Inactive as antitumour agents against lymphoid leukaemia (L 1210) | [142] |
| $R^2$ = Cl, Me, $NO_2$<br>$RR^1R^3R^4$ = H | Inactive as antitumour agents against lymphoid leukaemia (L 1210) | [142] |
| R = 4,6-Diamino-*s*-triazin-2-yl<br>$R^1$–$R^4$ = H | Potential dihydrofolic reductase inhibitor | [130] |
| $R^2$ = 4,6-Diamino-*s*-triazin-2-yl<br>$RR^1R^3R^4$ = H | Potential dihydrofolic reductase inhibitor | [130] |
| R = Br, I<br>$R^1R^2$ = H<br>$R^3$ = H, $CF_3$<br>$R^4$ = H, Me | Antisecretory activity | [164] |
|  (67) | | |
| R = H, Ac, COEt, Bz, $COCH_2CHMe_2$, $CO(CH_2)_2CO_2H$, CONHEt, CONHBu<br>$R^1$–$R^5$ = H, Cl | Muscle-relaxant, tranquilising, sedative, hypnotic and anti-secretory activity | [165] |
|  (68) | | |
| X = O, NH | Potential dihydrofolic reductase inhibitors | [166] |

Table 3.3. BIOLOGICAL PROPERTIES OF 3-AMINO- AND 3-HYDROXY-1,2,3-BENZOTRIAZIN-4(3*H*)-ONES

| *Compounds* | *Summary of biological properties* | *References* |
|---|---|---|
|  (69) | | |
| R = H, $CO_2Et$, $CO_2CH_2CH{=}CH_2$, $SO_2Me$, $SO_2Ph$<br>$R^1$ = H, Me, Et, allyl, benzyl, $CO_2Et$, $CH_2CO_2Et$<br>$R^2$ = H, Me, Ac, acylamino, OH, OAlk, halogen, $SO_2NH_2$ (in various positions) | Wide-ranging CNS effects | [167–169] |
| R = $CO_2(CH_2)_2OH$<br>$R^1R^2$ = H | Effect on EEG and anaesthesia | [170] |
|  (70) | | |
| R = H, Me | Analgesic and antipyretic activity | [167, 171, 172] |
|  (71) | | |
| R = H, $NO_2$<br>$R^1$ = H, $NO_2$, Cl, $Cl_2$ (various positions) | Bacteriostatic activity | [173] |
|  (72) | | |
| R = Cl, $NO_2$<br>$R^1$ = 5-nitro-2-furyl, $C_6H_4CF_3$-*p* | Bacteriostatic activity | [173] |
|  (73) | Anti-diarrhoeal activity; counteracts symptoms of poisoning by psychotropic drugs; analgesic and chemotherapeutic activity | [174] |

In none of the benzotriazines listed in *Tables 3.1–1.3* is there any evidence for involvement of covalent bonding between the benzotriazinone and its receptor, although in some of the studies this was the primary objective. The benzotriazinones (58; R = H or OH, R' = Et) were designed as photo-affinity agents on the assumption that they would complex reversibly with the active site of $\alpha$-chymotrypsin [105]. It was intended that photolytic degradation of the triazinone within the enzyme–inhibitor complex would lead to the generation of a reactive radical or ionic intermediate which could covalently react with components of the enzyme active site. This effort was frustrated by the photostability of the benzotriazinones [105, 147]. Moreover, the same triazinones have been recently shown to be neither substrates nor (reversible) inhibitors of the enzyme; evidently they do not productively bind to the active site [51].

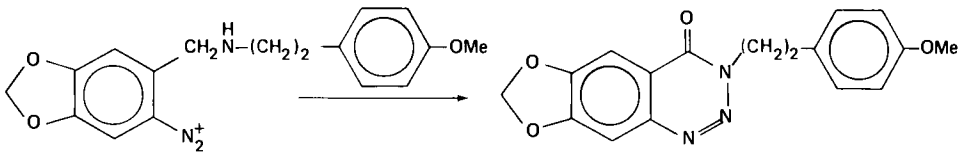
3-(1-Adamantyl)-1,2,3-benzotriazin-4(3*H*)-one (74) combines a virostatic adamantylamine fragment with the potential covalent bonding benzotriazinone moiety. No biological results have been published on this compound and its degradation products [144, 145].



(74)

The morpholinobenzotriazinone (70; R = H), for which a wide range of CNS effects have been claimed [167, 171, 172], can be estimated colorimetrically in tissue fluids by exploiting its acidic decomposition and coupling with *N*-(1-naphthyl)ethylenediamine (Bratton–Marshall Reagent) [167].

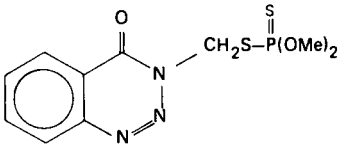
An observation of mechanistic interest was noted during an investigation of synthetic routes to the alkaloid lycorine [175]. An attempted Pschorr cyclisation to forge a C–C bond between the two phenyl rings of the diazonium salt (75) led to the oxidized product, the benzotriazinone (76). This surprising reaction is worthy of further study.



(75) (76)

To conclude this section, brief mention should be made of a series of insecticidal, parasiticidal and fungicidal benzotriazinones. In all these cases, the benzotriazinone group merely serves as a carrier for the biologically active moiety.

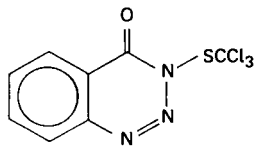
The organophosphorus insecticide, *O,O*-dimethyl *S*-[(3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl)methyl]phosphorodithioate (Guthion) (77), exhibits toxicological effects typical of cholinergic drugs in mice, rats and guinea-pigs [176]. Atropine raises the $LD_{50}$ dose to about twice its normal value.



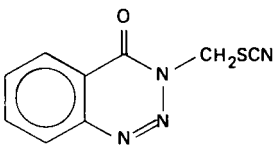
(77) Guthion

The insecticide is believed to be metabolised to an active anticholinergic oxo-analogue [176–178] (cf. similar activation of parathion and malathion): like most anticholinesterases of this type, its toxicity can be effectively countered by pyridine-2-aldoxime [179]. Both Guthion and its diethyl analogue are inhibitors of mitochondrial NADH-oxidase [180].

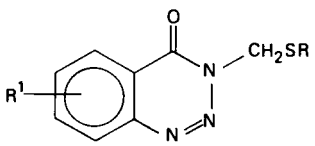
Other sulphur-containing substituted benzotriazinones include the nematocidal 3-trichloromethylthio-derivative (78) [181, 182], the fungicidal thiocyanomethyltriazinone (79) [183] and alkylthiomethyltriazinones (80) [184], and the insecticidal and antimicrobial thiosulphites (81) [185].



(78)

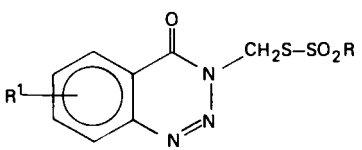


(79)



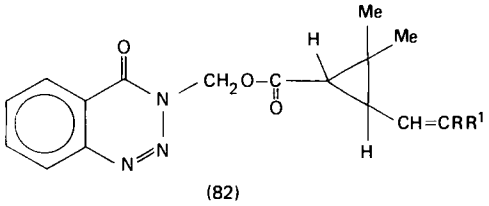
(80)

R = Lower alkyl
$R^1$ = Cl, $Cl_2$, $Cl_3$



(81)

R = Alkyl, Ar, aralkyl
$R^1$ = H, Cl, SMe



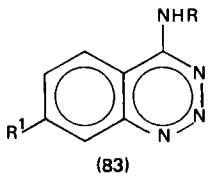
(82)

$RR^1$ = Straight or branched chain alkyl

Finally, the pyrethrum-inspired benzotriazinones (82) show insecticidal activity [186]. Although useful as agricultural chemicals, none of these latter benzotriazinones have apparently been examined for medicinal use.

## 4-AMINO- AND 4(3*H*)-IMINO-1,2,3-BENZOTRIAZINES

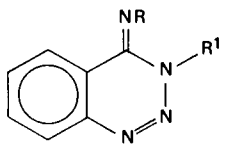
Grundmann and Ulrich [187] prepared a range of 4,7-disubstituted 1,2,3-benzotriazines (83a–e) and noted that 4-hydroxylamino-7-methoxy-1,2,3-benzotriazine (83e) possessed adrenergic blocking activity similar to that of apresoline, but was only 1/30 as active.



(83)

| | R | $R^1$ |
|---|---|---|
| (a) | H | H |
| (b) | $NH_2$ | H |
| (c) | $NH_2$ | Cl |
| (d) | OH | Cl |
| (e) | OH | OMe |
| (f) | Benzyl | H |
| (g) | Phenethyl | H |
| (h) | Ph | H |
| (i) | $C_6H_4Cl$–*o* | H |
| (j) | $C_6H_4NO_2$–*o* | H |
| (k) | $C_6H_4NO_2$–*m* | H |
| (l) | $C_6H_4NO_2$–*p* | H |

Other related 4-aralkyl- and 4-arylamino-1,2,3-benzotriazines (83f–l) and some 3-substituted 4(3*H*)-iminobenzotriazine counterparts (84a–e) have

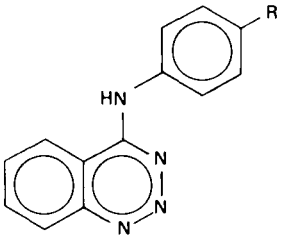


(84)

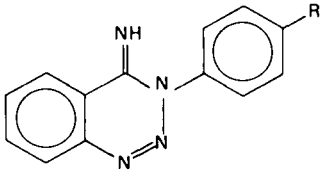
| | R | $R^1$ |
|---|---|---|
| (a) | H | Benzyl |
| (b) | H | Ph |
| (c) | Ph | Pr |
| (d) | Ph | $Pr^i$ |
| (e) | $C_6H_4Cl$–*o* | Pr |

been screened for tumour-inhibitory activity (against leukaemia L 1210) but found to be inactive [142].

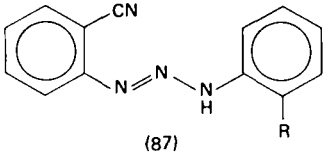
Similar inactivity was displayed by the benzotriazines (85) and (86). On the other hand, the *o*-cyanophenyltriazene (87) which is also a 'masked' diazo compound is modestly active against L 1210, P 388 (lymphocytic leukaemia), and human epidermoid carcinoma of the nasopharynx (H. Ep.-2) [142]. Its mode of action is as yet unknown, but it is possibly acting as an irreversible dihydrofolic reductase inhibitor [130].




(85)




(86)

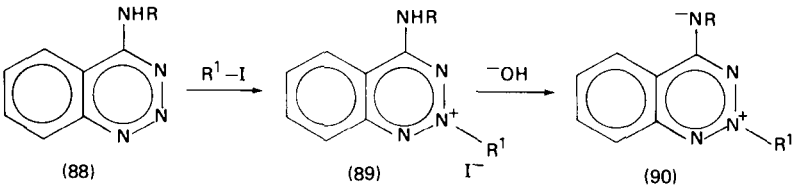



(87)

R = 4, 6—Diamino—*s*—triazin—2—yl

## 1,2,3-BENZOTRIAZINIUM COMPOUNDS

Alkylation of substituted 4-amino-1,2,3-benzotriazines (88) with alkyl iodides [126, 188] yields 2-alkyl-1,2,3-benzotriazinium iodides (89) with pharmacological activity [189].




(88) (89) (90)

R = Ph, *p*-tolyl, benzyl, phenethyl

R' = Me, Et, Pr, Pr', Bu, pentyl

In each of the four series with modified amino substituents, the potencies of the compounds (estimated on the frog rectus abdominis and chick biventer cervicis preparations) increased with an increasing number of carbon atoms in the 2-alkyl side-chain up to a maximum with the *n*-butyl iodides (*Figure 3.3*). This was unexpected, since replacement of methyl

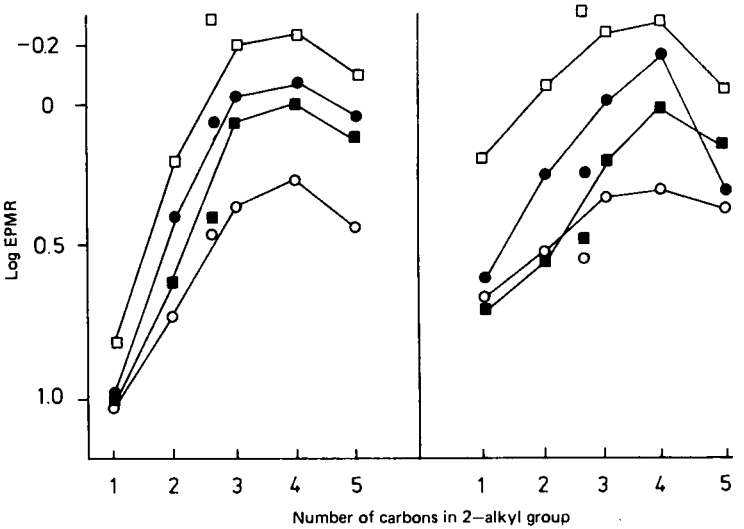



*Figure 3.3. Plot of activity of 2-alkylbenzotriazinium iodides (89) towards frog rectus abdominis (on the left) and chick biventer cervicis (right) against number of carbon atoms in the 2-alkyl side chain. Activity is expressed as the logarithm of the equipotent molar ratio (EPMR) relative to 4-anilino-2-*n*-propyl-1,2,3-benzotriazinium iodide (log EPMR = 0). The values for the i-propyl compounds have been inserted to the left of those for the n-propyl isomers* [189]. (●) *R = Ph.* (□) *R =* p-*Tolyl.* (○) *R = Benzyl.* (■) *R = Phenethyl*

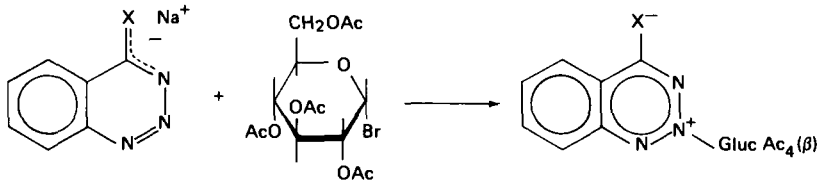
groups in quaternary ammonium compounds by larger alkyl groups almost invariably leads to a reduction in stimulating activity and frequently to the appearance of antagonist activity [190–192]. Responses to the tissues were not abolished by tubocurarine (i.e. the site of action is not at acetylcholine receptors). A remarkable feature of all the compounds is their high chloroform/water partition coefficients [189], and there appears to be a relationship between lipid solubility and biological activity in the series.

Neuromuscular-blocking activity has also been demonstrated in the 2-alkyl-1,2,3-benzotriazinium iodides (89) [193]. All the compounds caused an increase in the twitch height of the rat diaphragm twitch response,

followed by blockade–this blocking activity is qualitatively comparable with that of quinine and quinidine. Other puzzling but wide-ranging biological properties include a quinidine-like antiarrhythmic activity on isolated hearts from guinea-pigs and rabbits, and local anaesthetic activity comparable with that of lignocaine. In addition, certain derivatives possess anticholinesterase activity (about 1/1000 that of physostigmine); antagonism seems to be both competitive and non-competitive [194]. Striking similarities are apparent between the crystal structure of the iodide (89; R = Ph, R$^1$ = Pr) [188, 195] and quinidine [196], and it appears that the 2-alkyl-1,2,3-benzotriazinium iodides could comfortably fit a quinidine receptor, if this is indeed their mode of action.

The 2-alkylbenzotriazinium iodides (89) undergo dissociation into insoluble, highly coloured and biologically inert betaines (90) at slightly alkaline pH values [126]. This property severely limits the range of tissue preparations against which the compounds may be screened [189]. It would be interesting to examine the alkylation of 4-alkyl-, 4-aralkyl- or 4-aryl-1,2,3-benzotriazines since these triazines should afford 2-alkiodides with structural features which prohibit betaine formation: such triazinium compounds would be cationic at high pH values and could have interesting pharmacological activities.

Other 1,2,3-benzotriazinium betaines have been obtained from the alkylation [137] and glycosidation [138] of the sodium salt of 1,2,3-benzotriazin-4(3*H*)-one (91a). The product from (91a) and 2,3,4,6-tetra-*O*-acetyl-$\alpha$-D-glucopyranosyl bromide (92) was mainly the betaine (93a) together with minor amounts of the *N*(3)- and *O*-substituted isomers [138].

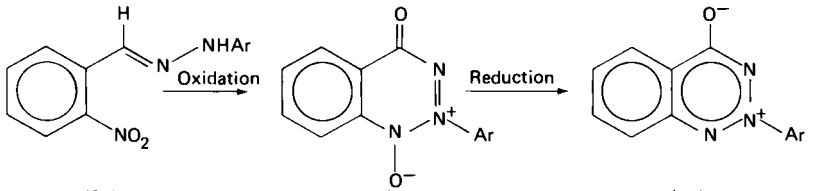



(91) (92) (93)

(a) X = O
(b) X = S

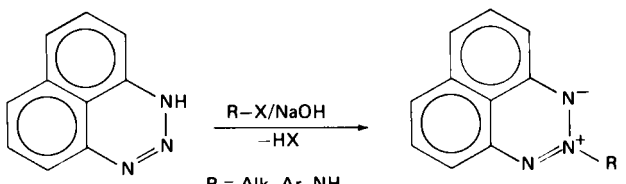
A similar betaine (93b) was obtained from 4-mercapto-1,2,3-benzotriazine (91b): removal of blocking acetyl groups was accomplished under alkaline conditions. These novel glycosides are worthy of biological examination.

Interesting betaines are formed in the oxidative transformation of arylhydrazones of *o*-nitrobenzaldehyde (94). This reaction was the subject of controversy [197] until Kerber [198] showed the products to be 2-aryl-1,2,3-benzotriazinium oxides (95): on reduction, they yield betaines (96) identical with the products formed by direct arylation of (91a) with diaryliodonium salts [139]. However, unlike the analogous alkylations, no isomeric *N*- or *O*-aryl derivatives are produced.



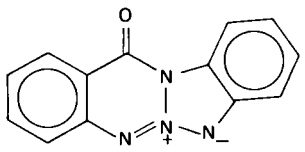

(94) (95) (96)

Other unusual 1,2,3-triazinium compounds which it is appropriate to mention at this point include the dipolar naphthotriazines (98; R = Alk, Ar, $NH_2$) prepared by direct alkylation [199–201], arylation [202, 203] or amination [204, 205] of 1*H*-naphtho[1,8-*de*]triazine (97).

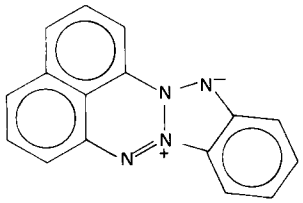



(97) (98)

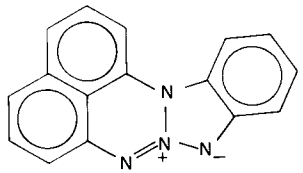
Deoxygenation of *o*-nitrophenyl-1,2,3-triazines with triethyl phosphite provides a smooth route to the dipolar 1,2,3-benzotriazinone (99) [206], and the unusual pentacyclic dipolar triazines (100) and (101) [202, 203]. These latter two compounds and their *C*-substituted cogeners are the only examples of 1,2,3-benzotriazines annelated at the *b*, *d* and *e* bonds.




(99)



(100)



(101)

Although structures (98–101) are not formally cationic in the manner of the 2-alkyl-1,2,3-benzotriazinium iodides (89), they present an interesting combination of polar and hydrophobic groups which could give them versatile binding potential: their biological properties, however, do not appear to have been evaluated.

## AZINO- AND AZOLO-*c*-[1,2,3]BENZOTRIAZINES

2,4-Diaminopyrimidines and 2,4-diamino-*s*-triazines have found widespread use as medicinal agents [207]. They complex reversibly with H-bonding or ionic sites (to which the diamino-hetero fragment binds) and to an adjacent hydrophobic region at the active site of dihydrofolic reductase [208]. 4-Amino-2(2*H*)-imino-*s*-triazino[1,2-*c*][1,2,3]benzotriazine (43) and its derivatives were designed with structural groups complementary to these features of the surface of the enzyme and should bind to the active site (possibly as depicted in *Figure 3.4*). Although triazine (43) and its analogues decomposed heterolytically and homolytically depending on the nature of the substrate [101] (see also p. 219), no tumour-inhibitory activity was discerned against leukaemia L 1210 [142]. An aryl radical generated from (43) by homolytic fission of the triazine ring would be an ideal species to react covalently with the enzyme hydrophobic area.

The diazonium chlorides (102), which are the ring-opened forms of the *s*-triazinobenzotriazines (43), are also inactive against L 1210 [142] whereas the diazonium fluoroborate (103) prolonged the life of mice implanted with L 1210 by 157% at 1.25 mg/kg and produced 5 out of 6 cures in Dunning leukaemia (ascites) at 0.63 mg/kg [209]. In this exam-

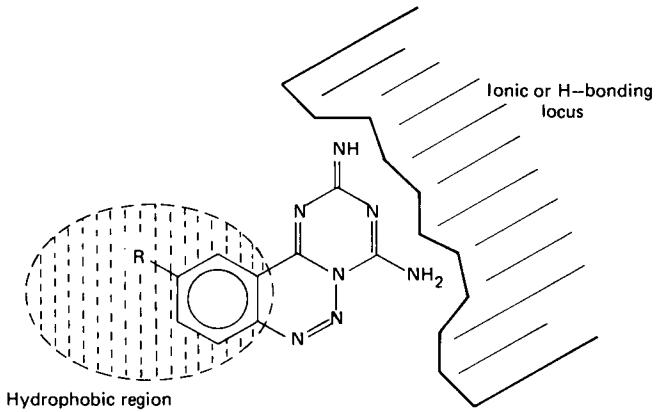
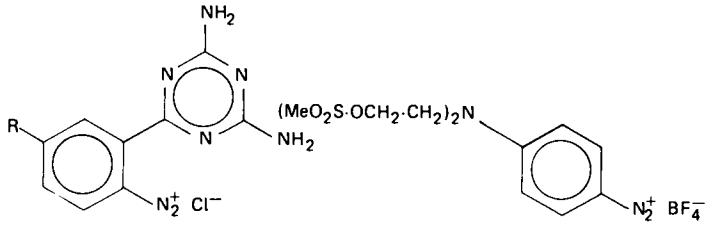



*Figure 3.4. Possible mode of binding between the active site of dihydrofolic reductase and 4-amino-2(2H)-imino-s-triazino [1,2-c][1,2,3]benzotriazines*

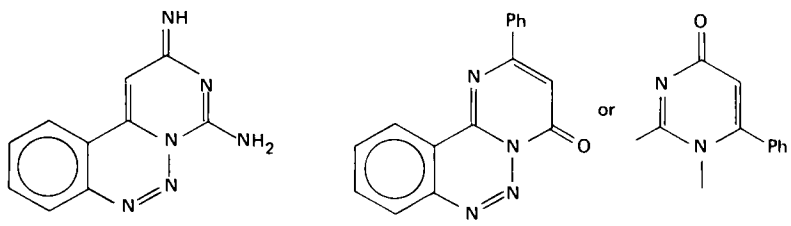
ple, however, it is the alkylating fragment rather than the diazonium group which is probably the cytotoxic species.



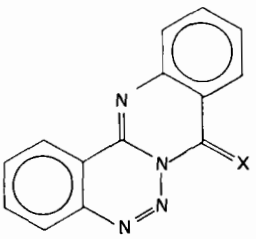

(102)
R = H, Me, Br

(103)

Attempts to prepare the potentially useful pyrimido[1,6-*c*][1,2,3]-benzotriazine (104) have not been successful [166] and other 1,2,3-benzotriazines fused to an azino ring at the *c* bond which have not been evaluated biologically include the pyrimidobenzotriazines (105 a or b)



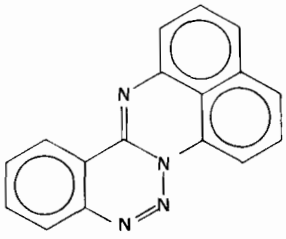

(104) (105a) (105b)



(106)

(a) X = NH

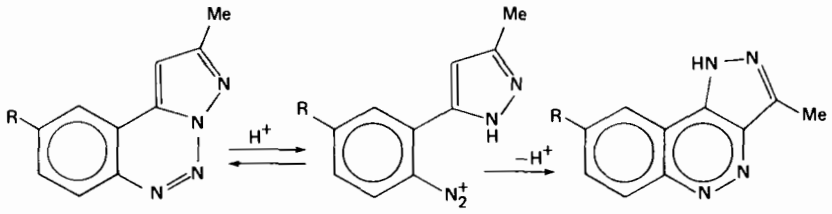
(b) X = O



(107)

[210], the quinazol-imine (106a) [125] and quinazol-one (106b) [115] and the pentacyclic triazine (107) [211].

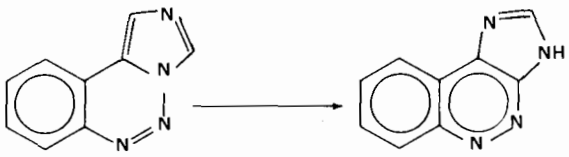
Isomerisation of the pyrazolo[1,5-*c*][1,2,3]benzotriazines (108) in warm acid proceeds via the ring-opened diazonium ions (109) which recyclise irreversibly by *C*-coupling to form the isomeric pyrazolo [4,3-*c*]cinnoline derivatives (110) [122, 212]. The reaction has affinities with the diazoaminobenzene – *p*-aminoazobenzene rearrangement.



(108) (109) (110)

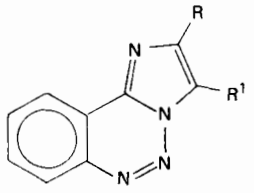
R = H, OMe, OEt

The requirement in both these rearrangements for an activated aromatic nucleus susceptible to electrophilic attack at carbon, is also met in the imidazo[1,5-*c*][1,2,3]benzotriazine (111) [213]: possibly this compound could also rearrange (to the imidazocinnoline (112)).



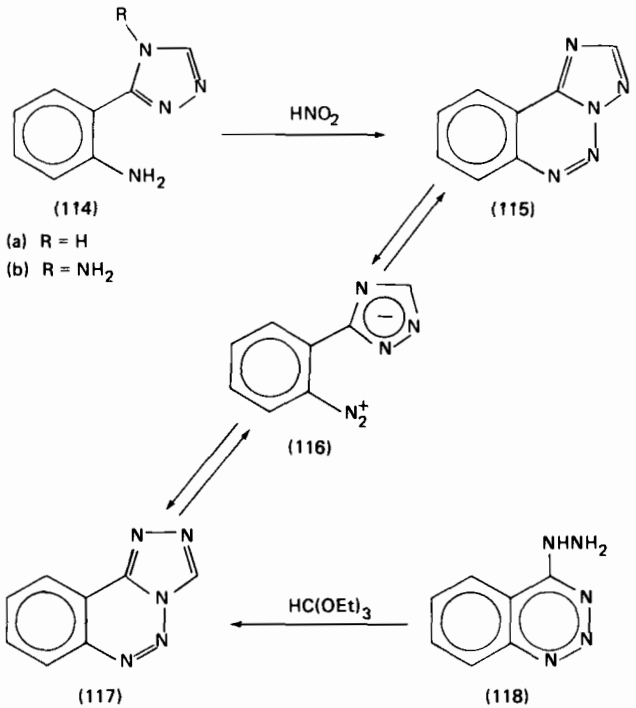
(111) (112)

No such rearrangement can occur in the isomeric imidazo[1.2-*c*] [1,2,3]-benzotriazine (113; R = R' = H) [213]. Interestingly, one derivative of this ring-system (113; R = Me, R' = Ac) inhibits measles virus in HeLa cell culture at a concentration of 0.52 $\mu$g/ml [214] but has no antibacterial activity [215].



(113)

Diazotisation of the o-aminophenyl-*s*-triazole (114a) with one equivalent of nitrous acid [216], or the *N*-aminotriazole (114b) with two equivalents [217] gave a triazolobenzotriazine which might be either triazolo[1,5-*c*][1,2,3]benzotriazine (115), or the [4,3-*c*] isomer (117).




(114)

(a) R = H

(b) R = $NH_2$

(115)

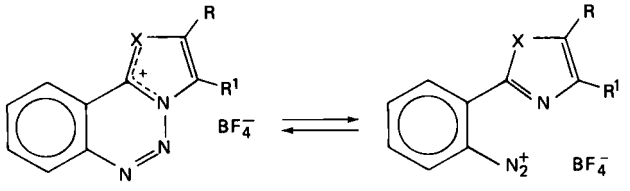
(116)

(117)

(118)

The observation that the same product is obtained by heating 4-hydrazinobenzotriazine (118), with triethyl orthoformate [216] does not necessarily imply that the [4, 3-*c*] arrangement (117) is preferred, since Dimroth rearrangement to the thermodynamically more stable isomer can occur via a diazonium species (116) [135, 218]. There are ample precedents in the literature of cyclic 1,2,4-triazoles to confidently predict that isomer (115) with $N(1)$ of the triazole ring at the bridgehead position would be more stable than isomer (117) with $N(4)$ at the ring junction [219].

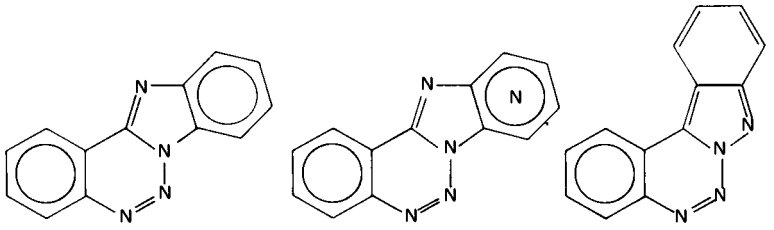
The oxazolo[3,2-*c*]- and thiazolo[3,2-*c*][1,2,3]benzotriazinium salts (119a and b respectively) exist in equilibrium with their ring-opened forms (120a and b) [220].



(119) (120)

(a) X = O; R = Me, Ph, $C_6H_4Br$–*p*; $R^1$ = H
(b) X = S; R = H; $R^1$ = Me, Ph, $C_6H_4Br$–*p*

Other azolo-*c*-[1,2,3]benzotriazines which have been prepared (but not examined biologically) include derivatives of benzimidazo[1,2-*c*]-[1,2,3]benzotriazine (121) and some heteroaromatic analogues (122) [107, 221–223], and the indazolobenzotriazine (123) [224].

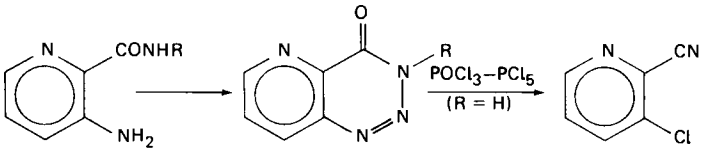


(121) (122) (123)

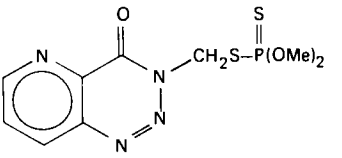
## AZINO-*d*-[1,2,3]TRIAZINES

The synthesis and chemistry of azino-*d*-triazines closely parallels that of the benzotriazine series.

Nitrosation of 3-aminopyridine-2-carboxamide (124a) with amyl nitrite in acetic acid gives pyrido[3,2-*d*][1,2,3]triazine-4(3*H*)-one (125a) [225]. Degradation of the pyridotriazinone in $POCl_3$–$PCl_5$ yields the chloronitrile (126), a reaction with a precedent in the 1,2,3-benzotriazine series [226].




(124) (a) R = H (b) R = OH (125) (126)

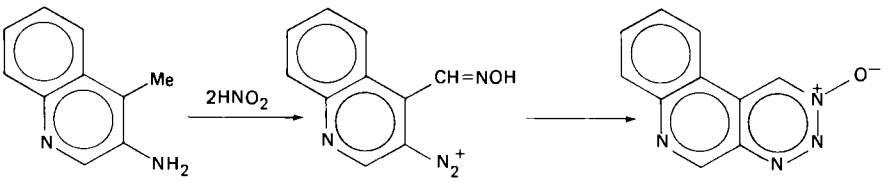



(127)

The corresponding *N*-hydroxypyridotriazinone (125b) was prepared in a programme of research on schistosomicidal agents, but was inactive [227].

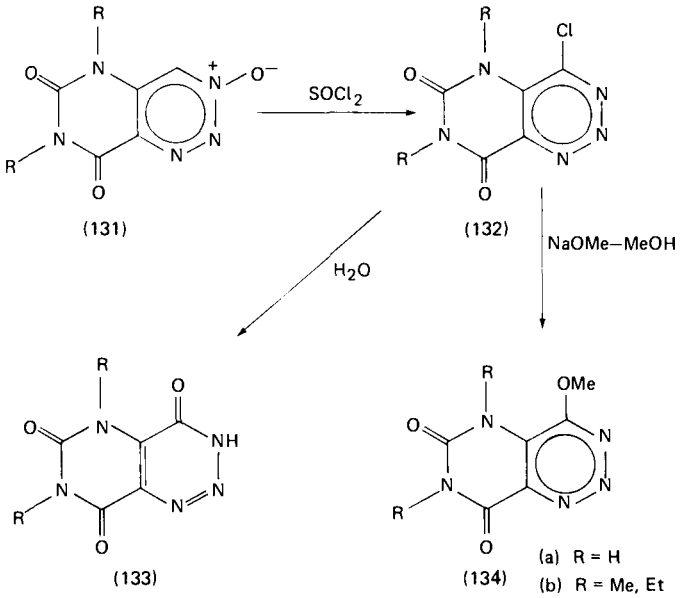
The aza-analogue of Guthion (127) possesses acaricidal and nematocidal activity [228].

Diazotisation of 3-amino-4-methylquinoline (128) with two equivalents of nitrous acid gives an intermediate believed to be the diazo-aldoxime (129) which cyclises spontaneously to the triazine-*N*-oxide (130) [229].



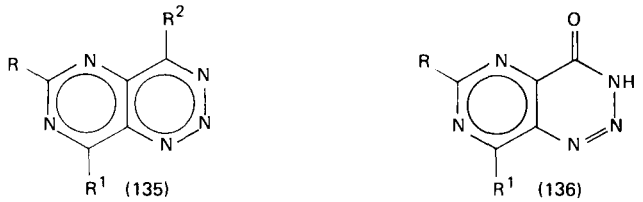

(128) (129) (130)

Similar diazotisations afford a convenient route to the unsubstituted dioxopyrimido[5,4-d][1,2,3]triazine-*N*-oxide (131a) [230] and its di-*N*-alkyl homologues (131b) [231].

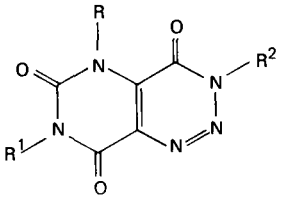



The conversion of the di-*N*-alkyltriazine oxides (131b) to chlorotriazines (132b) is a reaction apparently without precedent in the 1,2,3-benzotriazine-*N*-oxide field, and subsequent transformations of the chlorotriazines to the triazinones (133b) and methyl ethers (134b) yield a useful range of compounds with diuretic, anti-inflammatory and antibacterial activity [232].

Other pyrimido[5,4-*d*][1,2,3]triazines which have been examined for biological activity include the structural types (135–137) which exhibit cardiovascular, spasmolytic and diuretic activity, and CNS effects [233, 234].

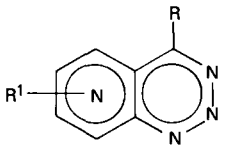



$RR^1$ = Range of cyclic and non-cyclic *t*-amino groups
$R^2$ = Cl or *t*-amino groups



(137) $RR^1$ = H, Me, benzyl
$R^2$ = Me, Et, benzyl

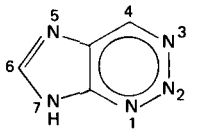
To conclude this section, mention should be made of the amination-oxidation route to bicyclic 1,2,3-triazines (see p. 221) which has been successfully applied to the synthesis of azino-*d*-triazines of general structure (138) [131, 235].



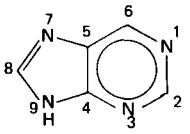
(138)

## IMIDAZO[1,2,3]TRIAZINES (2-AZAPURINES)

Structural analogues of naturally occurring purines are potential substrates for (or inhibitors of) many of the enzymes controlling the cellular metabolism of purine nucleotides [236]. Substitution of a purine CH by N has been a fruitful area of research into purine antimetabolites; such a substitution in the 2-position of purine affords 7*H*-imidazo[4,5-d][1,2,3]triazine (139). The *Chemical Abstracts* numbering for this ring-system is as indicated, but derivatives of imidazotriazine are commonly referred to as 2-azapurines to emphasise their relationship to purine (140).



(139)

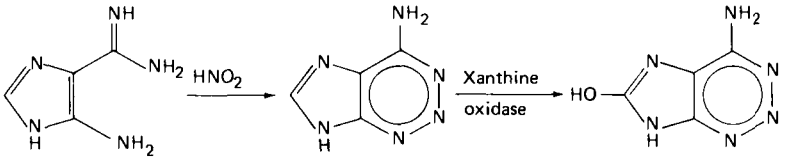


(140)

Aza-substitution in the purine 2-position might be expected to have a more profound effect than the corresponding 8-substitution (to give 8-azapurines [237]) since the products now become masked diazo derivatives with all that implies in terms of potential covalent reactions.

*2-Aza-adenine*

Woolley and Shaw first synthesised 2-aza-adenine (142) by nitrosation of 5-aminoimidazole-4-carboxamidine (141) [238] and demonstrated its inhibitory activity against *Lactobacillus brevis* and *L. arabinosus*, other lactic acid bacteria, and yeast; it was transformed by xanthine oxidase to 8-hydroxy-2-aza-adenine (143) [239].




(141) (142) (143)

2-Aza-adenine

The toxicity of 2-aza-adenine to mice can be antagonised by adenine or hypoxanthine [238] and cytotoxicity towards sarcoma 180 cells in tissue culture blocked by adenine [240]. Anti-tumour activity against H. Ep-2 cells [241, 242] and antiviral activity against tobacco mosaic virus has been reported [243]. Other efforts to demonstrate the involvement of 2-aza-adenine in purine metabolism have taken the form of theoretical calculations [244, 245] and microbiological studies [246–248].

*2-Aza-adenine nucleosides*

2-Aza-adenine is converted to 2-aza-adenosine triphosphate by incubation with human erythrocytes at pH 7.4 [249]: this triphosphate is itself hydrolysed by erythrocyte membrane ATPase [249, 250]. In addition, 2-aza-adenosine strongly inhibits platelet aggregation in man and produces vasodilation in the human forearm; these responses may be mediated by similar receptors [251]. More recently, 2-aza-adenine and 2-aza-adenosine have been shown to be potent inhibitors of adenine phosphoribosyltransferase [252]. The 2-position is probably not critically involved in binding to the enzyme [253].

The development of synthetic methods to prepare 2-aza-adenine nucleosides was prompted by the observation that adenine-1-oxide (144a) may be hydrolysed by acid to 5-aminoimidazole-4-carboxamidoxime (145a) which may be further converted to 2-aza-adenine-1-oxide (146a) by nitrosative ring closure [254, 255]. Oxidation of 2-aza-adenine (150a) with hydrogen peroxide–acetic acid afforded the same 1-oxide (146a) [255]: it was envisaged that this 1-oxide and 2-aza-adenosine-1-oxide (146b) could act as pro-drugs being reduced to the active 2-azapurines in vivo.

Montgomery and Thomas [5, 256] have adapted the above procedures to achieve a notable five-step conversion of adenine nucleosides to 2-aza-adenine nucleosides. Benzylation of adenine oxides (144a–h) yields *N*-benzyloxyadenines (147a–h) which smoothly ring-open and deformylate in methanolic ammonia. Catalytic hydrogenation of the resultant *N*-benzyloxyamidines (148a–h) affords 5-aminoimidazole-4-carboxamidine and its nucleosides (149a–h) which cyclise on nitrosation to 2-aza-adenine and its nucleosides (150a–h).

The cytotoxic properties of the nucleosides against H.Ep.-2 cells are recorded in *Table 3.4.* 2-Aza-adenosine is the most active. In addition 2-aza-adenosine given at dose levels of 75–400 mg/kg (on day one only) increased the life-span of $BDF_1$ mice inoculated with $10^5$ leukaemia L 1210 cells by 30–35%. None of the other nucleosides were active in this test system [5].

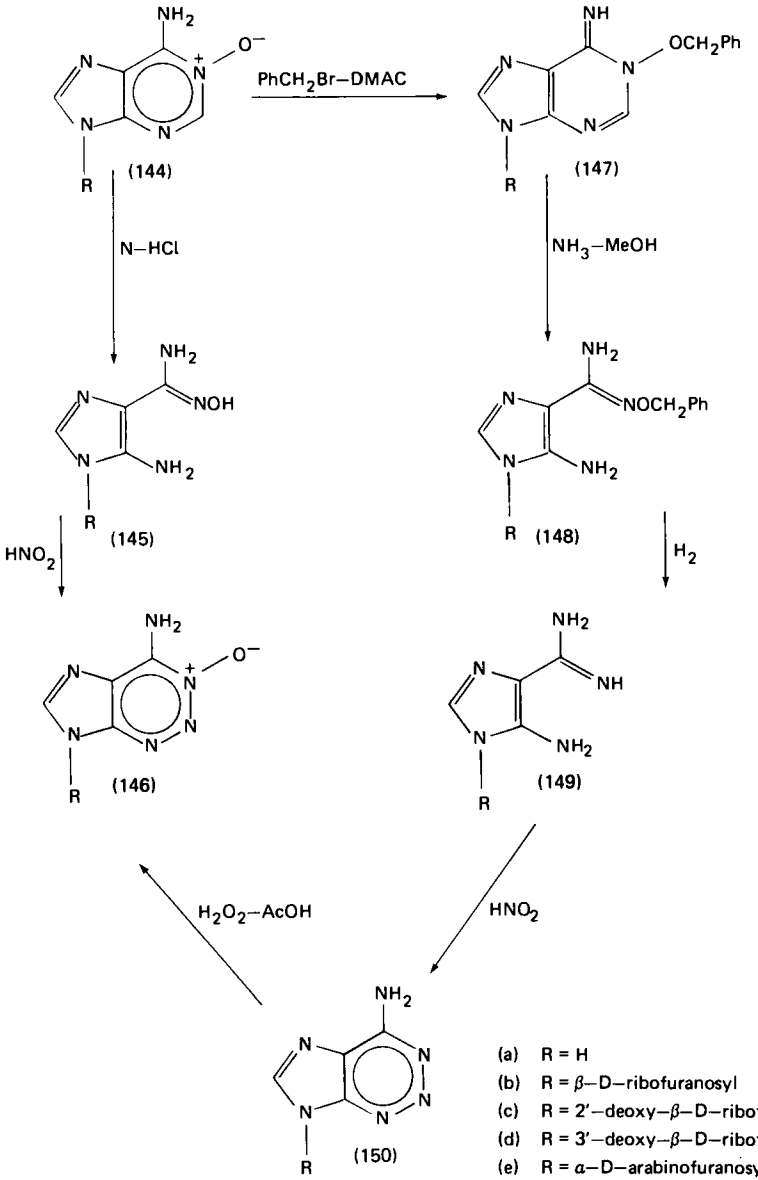
Table 3.4. CYTOTOXICITY OF 2-AZA-ADENINE AND ITS NUCLEOSIDES AGAINST H.Ep.-2[5, 257]

| *Structure* | *Name* | $ED_{50}(\mu M)$[a] |
|---|---|---|
| 150a | 2-aza-adenine | 4.0[b]–10[c] |
| 150b | 2-aza-adenosine | 0.2[c]–0.22[b] |
| 150c | 2′-deoxy-2-aza-adenosine | $<2.0$[b] |
| 150d | 3′-deoxy-2-aza-adenosine | 80[c] |
| 150e | 9-α-D-arabinofuranosyl-2-aza-adenine | $>200$[c] |
| 150f | 9-β-D-arabinofuranosyl-2-aza-adenine | $>100$[b] |
| 150g | 9-β-D-xylofuranosyl-2-aza-adenine | $>100$[b] |
| 150h | 9-cyclopentyl-2-aza-adenine | 49[b] |

[a] Concentration required to inhibit the growth of treated cells to 50% of that of untreated controls

[b] Reference [5].

[c] Reference [257].




(a) R = H
(b) R = β–D–ribofuranosyl
(c) R = 2′–deoxy–β–D–ribofuranosyl
(d) R = 3′–deoxy–β–D–ribofuranosyl
(e) R = α–D–arabinofuranosyl
(f) R = β–D–arabinofuranosyl
(g) R = β–D–xylofuranosyl
(h) R = cyclopentyl

Recent work on the effects of these nucleosides against a series of H.Ep-2 cell lines lacking specific enzymes shows that 2-aza-adenine is inactive only against the resistant line lacking adenine phosphoribosyltransferase; it must therefore be activated by conversion to 2-aza-adenylic acid [257]. Other biochemical explanations for the mode of action of these nucleosides, including possible incorporation into nucleic acids, have been advanced [257]. No evidence of a biological role for a ring-opened diazonium species has been found. Although at acidic pH the 1,2,3-triazine ring could be cleaved–and ideal conditions for ring-cleavage could be created if the 2-aza-adenine nucleosides were incorporated intact into nucleic acids–in alkaline conditions it is the imidazole ring of 9-cyclopentyl-2-aza-adenine which is ring-opened [5] (see p. 218).

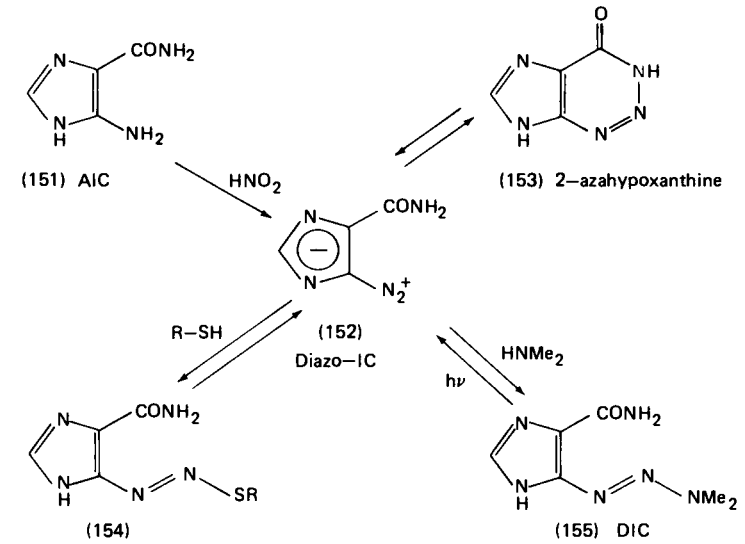
*2-Azahypoxanthine*

5-Aminoimidazole-4-carboxamide (151; AIC or AICA) was isolated from *E.coli* cultures inhibited by sulphonamides by Stetten and Fox [258, 259] who confirmed its amine character by diazotisation to yield an unidentified product. This product was later identified as 2-azahypoxanthine (153). The initial product of the diazotisation of AIC is, however, the diazoimidazole (152; Diazo-IC or Diazo-ICA) which can be independently cyclised to 2-azahypoxanthine in acidic, basic or neutral conditions [260].

Diazo-IC behaves like a typical aromatic diazonium compound, and, in spite of the competing intramolecular cyclisation, couples with aromatic substrates [261], dialkyl- and aralkylamines [261–263], monoalkyl- and monoarylamines [263–265], hydrazines [266], amidines [267], thiols [268–270] and other nucleophiles. Some of the chemical and biological properties of Diazo-IC have been reviewed [271].

Interpretation of biological results concerning Diazo-IC and 2-azahypoxanthine is complicated by the possibility of interconversion of the two forms in vitro and in vivo. A compound claimed to be 2-azahypoxanthine, and with inhibitory activity against *L. brevis* and *L. arabinosus* that was competitively reversed by hypoxanthine [238], was probably a mixture of Diazo-IC and 2-azahypoxanthine [260]. With suitable precautions in administration Diazo-IC was shown to inhibit H.Ep.-2 cells in tissue culture [260], and Ehrlich ascites carcinoma in mice [260, 263]: 2-Azahypoxanthine was inactive in the H.Ep.-2 test and was generally less toxic to mice than Diazo-IC [260]. Both acyclic and cyclic compounds inhibit the growth of *Mycobacterium tuberculosis* [272].

Diazo-IC and two thioazo analogues (154a and b) are potent inhibitors of




(a) R = $CH_2$–$CH(NH_3^+)CO_2^-$
(b) R = $(CH_2)_2NH_2$
(c) R = $(CH_2)_2OH$
(d) R = $C_6H_4NO_2$–*p*
(e) R = $C_6H_4NH_2$–*o*

rat liver and cream xanthine oxidase [273]. The equipotent concentrations of Diazo-IC and allopurinol producing 50% inhibition of the enzyme after aerobic incubation for 10 min at 23°C are $8 \times 10^{-7}$ M and $2 \times 10^{-6}$ M respectively; 2-azahypoxanthine is much less active. Pre-incubation of Diazo-IC and its thioazo analogues in buffer (in which they are all converted to 2-azahypoxanthine) markedly reduces their activities [274]. Significantly, the activity of Diazo-IC is suppressed by cysteamine, reduced glutathione and cysteine (but not other amino acids) in a process strongly catalysed by metal ions. Possibly Diazo-IC reacts covalently with vital thiol groups on the enzyme [274].

2-Azahypoxanthine is the inhibitory entity against bovine kidney uricase, and Diazo-IC and the thioazo derivatives (154b–e) apparently merely act in a pro-drug role [275]. 2-Azahypoxanthine is also weakly inhibitory against hypoxanthine-guanine phosphoribosyltransferase whereas Diazo-IC is inactive [275]. The observation that Diazo-IC inhibits conversion of [$^{14}$C]glycine to hypoxanthine by pigeon liver homogenates may indicate a more fundamental interference with de novo purine synthesis [276].

In vitro activation of monoamine oxidase activity in rat liver homogenates [277] and calcium-dependent release of 5-hydroxytryptamine (5-HT) from rabbit platelets by Diazo-IC can be blocked by thiols [278, 279]. Diazo-IC can also potentiate hexobarbital hypnosis in mice by depressing hepatic metabolism of the drug. 2-Azahypoxanthine is inactive in this respect [280].

Diazo-IC and 2-azahypoxanthine are formed in the light-catalysed dissociation of 5-(3,3-dialkyl-1-triazeno)imidazole-4-carboxamides. One such compound, the dimethyltriazene (155; DIC, DTIC, NSC-45388 or Dacarbazine®), is a useful agent for the control of malignant melanoma and has inhibitory action against a range of tumour-systems [281]. The chemical and biological properties of DIC and related triazenoimidazoles have been comprehensively reviewed [271], and this work will merely re-emphasise the two major decomposition pathways of the drug.

Although DIC is stable in the dark in the solid state, (or) in phosphate buffer, aqueous ethanol, 0.1 N hydrochloric acid or lactated Ringer's solution, exposure of the solutions to light of varying wavelengths affords 2-azahypoxanthine [260, 262]. Analysis of DIC in biological fluids may be accomplished by light-initiated dissociation of the drug in the presence of the Bratton–Marshall reagent [282]. The initially formed Diazo-IC couples to form an azo-dye whereas 2-azahypoxanthine is unreactive [260].

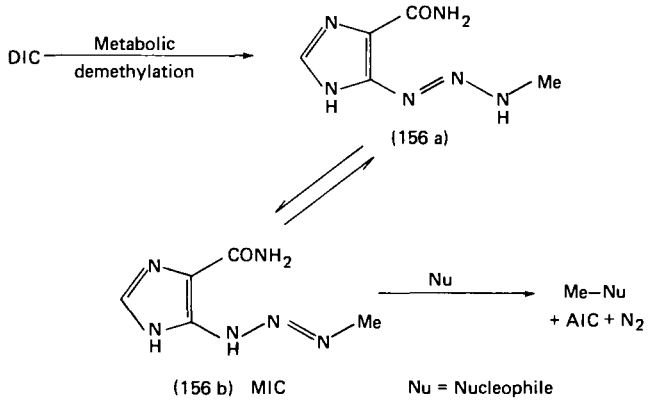
It has been suggested that DIC may be a transport form of Diazo-IC [263, 283] since DIC itself causes little loss of transplantability capacity in Ehrlich ascites cells whereas exposure to Diazo-IC considerably suppresses transplantability. However, there is no correlation between antitumour activity and rate of formation of Diazo-IC from a range of dialkyltriazenoimidazoles [263]. Both Diazo-IC and DIC suppress growth of *E.coli* B without causing cell lysis [284] and the inhibitory effect of Diazo-IC can be abolished by cysteine (but not by AIC or hypoxanthine). However, cysteine gives no protection against foetal abnormalities produced in rats by DIC [285]. At relatively high concentrations 2-azahypoxanthine inhibits *E.coli* B cell growth, and its effect can be abolished by hypoxanthine [284].

Pre-exposure of DIC to light markedly *decreases* its lethality to *B. subtilis* [286] (presumably because of rapid photo-conversion to inactive 2-azahypoxanthine), but exposure to light of cultures growing in the presence of DIC–conditions which would favour the continuous generation of Diazo-IC–dramatically *increases* the inhibitory effect compared with cultures growing in the dark. In addition, a DIC-resistant strain of *B.*

*subtilis* is also resistant to Diazo-IC. Evidently Diazo-IC is the inhibitory moiety in this test system [286].

DIC is also more lethal to Chinese hamster ovary cells and malignant human melanoma cells in culture in the presence of light [287–289], but with DIC labelled with $^{14}C$ at the imidazole 2-position radioactivity is incorporated into RNA and DNA regardless of the light conditions [287]. Interestingly, 2-azahypoxanthine was identified as a transformation product of DIC in Chinese hamster ovary cells even in the dark [289]; presumably it is a simple chemical degradation product (metabonate) in this case.

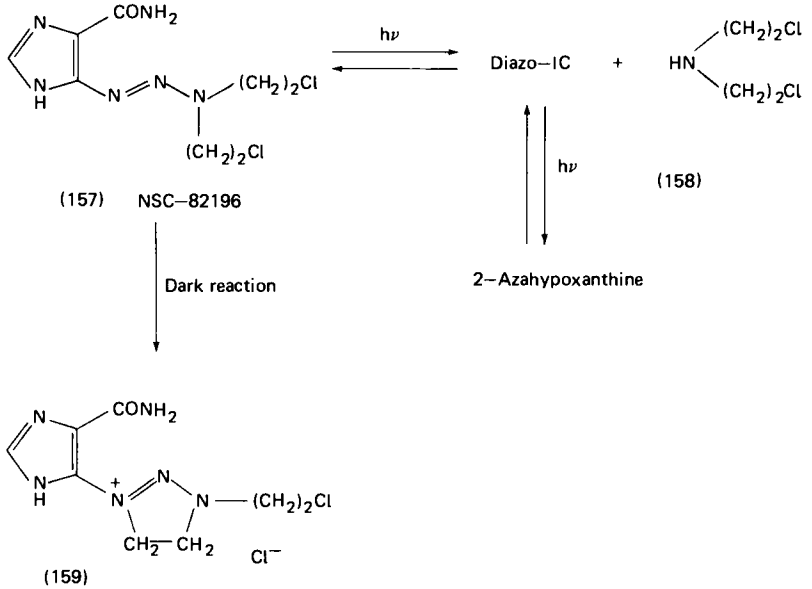
These results in in vitro systems are consistent with current theories regarding the mode of action of DIC: on exposure to light DIC undergoes photo-decomposition to dimethylamine and Diazo-IC which may interfere with purine metabolism or react with vital thiol groups in an obscure manner [284, 287–289]. In vivo (presumably in the dark), there is compelling evidence that DIC is metabolised by oxidative de-methylation to the unstable 5-(3-methyl-1-triazeno)imidazole-4-carboxamide (156; MIC) [264, 290–295].




The essential requirement for at least one methyl group in the tumour-inhibitory triazenes has been unequivocally established [292], and many of the inhibitory effects of DIC can be accounted for by the action of MIC [293]. MIC is unstable in physiological fluids in the dark and decomposes to yield AIC and a 'methylating fragment' which can methylate DNA [271, 293, 295, 296], notably N(7) of guanine residues. The consequences of this biochemical lesion (like those elicited by other alkylating agents) would be profound for the well-being of the cell.

The nature of the methylating species is the subject of controversy: it is certainly not diazomethane (or methylene) since the methyl group of MIC is transferred intact to nucleic acids [295]. Decomposition of a series of monoalkyltriazenoimidazoles follows first-order kinetics in water and methanol [264], and the identification of 2-butene from the spontaneous decomposition of 5-(3-t-butyl-1-triazeno)imidazole-4-carboxamide implies the intermediacy of a t-butyl carbenium ion. The involvement of a transient 'free' methyl carbenium ion from MIC in a biological environment, however, is highly questionable. Although this fugitive species may, in its MIC carrier form, survive transport from the major site of generation (the liver) to a distant target site in the nuclei of cells, once liberated as a methyl carbenium ion it would be swamped in the pool of cellular nucleophiles and not achieve any selectivity in its reactions. Reference [271] should be consulted for further discussion on the mode of action of DIC, MIC and other alkyltriazenes. A mechanistic study of the decomposition of monoalkyltriazenes has recently been reported [297], and the similarities between monoalkyltriazene decomposition and 'diazo migration' [35–38] should be noted (see p. 210).

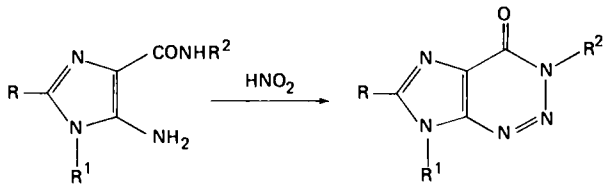
Before leaving the subject of tumour-inhibitory dialkyltriazenes, some properties of 5-[3,3-bis(2-chloroethyl)-1-triazeno]imidazole-4-carboxamide (157; NSC-82196) should be mentioned. This triazene displays



potent anti-leukaemic activity in murine tests [271, 298–300] and its cytokinetic effects against L 1210 cells in vivo have been compared with those of DIC [301]. In the light, NSC-82196 dissociates to form Diazo-IC (and thence 2-azahypoxanthine) and bis(2-chloroethyl)amine (158) [298, 299, 302, 303]. In vitro in the dark, and in vivo the major transformation product is the 1,2,3-triazolinium chloride (159) [302, 304]. As with DIC, elucidation of the exact mode of action of NSC-82196 must await more definitive chemical and biochemical examination [271].

### *2-Azahypoxanthine nucleosides*

Exploratory experiments on the diazotisation of substituted 5-aminoimidazole-4-carboxamides (160) indicated that cyclisation proceeded smoothly to the corresponding substituted 2-azahypoxanthines (161) [305–307].

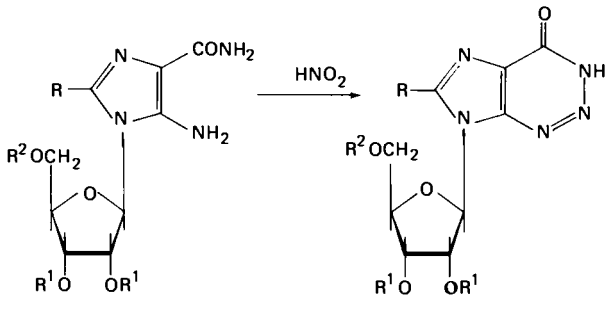



(160) (161)

Internally-compensated diazoimidazoles (cf. Diazo-IC) are not possible in these cases except when $R^1 = H$. The main adaptation necessary for nucleoside formation involves diazotising the appropriate aminoimidazole carboxamides (162) at −30°C in 6 N hydrochloric acid to avoid acidic hydrolysis of the glycosyl linkage [308]. By this means, 2-azainosine (163a), 2-azainosinic acid (163b) and the 2′,3′,5′-tri-*O*-acetyl derivative of 2-azainosine (163c) have been prepared [308, 309]. The p*K*a of 2-azainosine is more than two units lower than that of inosine [309].

2-Azainosine inhibits growth of parainfluenza virus cultured in KB cells, and 2-azainosinic acid and the triacetate (163c) inhibit herpes simplex virus. All three nucleosides show activity against KB, H.Ep.-2 and HeLa cells in tissue culture [309].

Bromination of the imidazole carboxamide nucleoside (162c) with *N*-bromoacetamide in anhydrous tetrahydrofuran affords the corresponding bromoimidazole carboxamide (162d) which can be cyclised to the



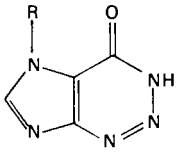
(162) (163)

(a) R = $R^1$ = $R^2$ = H
(b) R = $R^1$ = H, $R^2$ = $PO_3H_2$
(c) R = H, $R^1$ = $R^2$ = Ac
(d) R = Br, $R^1$ = $R^2$ = Ac

bromoimidazotriazine (163d) by nitrosation at −25°C [310]. Replacement of bromine by amino, azido, hydroxy, methoxy and thiol groups, and deacylation of the sugar residue, can be achieved by conventional chemical procedures [310]. Other 2-azahypoxanthine nucleosides can be prepared from 2-aza-adenine nucleosides by deamination with adenosine deaminase, or from hypoxanthine nucleosides by adaptation of the ring-opening and ring-closure sequence used for converting adenine nucleosides to 2-aza-adenine nucleosides [257]. In biological tests 2-azahypoxanthine, 2-azainosine and 2′-deoxy-2-azainosine were inactive against H.Ep.-2 cell lines lacking hypoxanthine-guanine phosphoribosyltransferase. In fact, 2-azahypoxanthine nucleosides are less promising as cytotoxic agents than the corresponding 2-aza-adenine nucleosides since they are apparently simply latent forms of 2-azahypoxanthine to which they are converted by inosine phosphorylase [257].

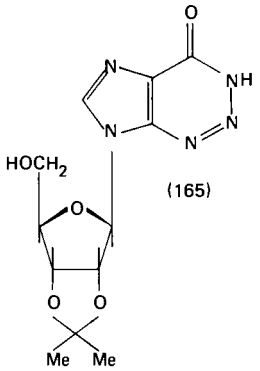
Other unusual 2-azahypoxanthine derivatives which have been prepared include 5-methylimidazo[4,5-*d*][1,2,3]triazin-4(3*H*)-one (164a) and the 5-$\beta$-D-ribofuranosyl analogue (164b) [308], and the isopropylidene derivative of the $\alpha$-anomer of 2-azainosine (165) [311].

Cyclisation of 5-diazoimidazole-4-thiocarboxamide (166) is reported to yield first the imino-thiadiazine (167) which then rearranges in aqueous ammonia to the mercaptotriazine (168). Methylation of the thiol gives an *S*-methyl derivative which is sensitive to nucleophilic displacement of methyl mercaptan [312] and can be converted directly into its 2′,3′,5′-tri-



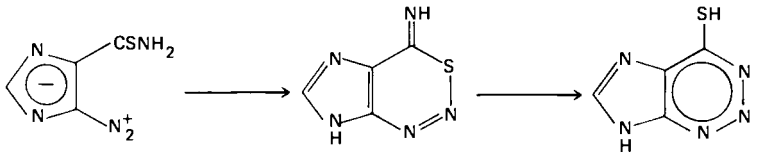
(164)

(a) R = Me
(b) R = β–D–ribofuranosyl



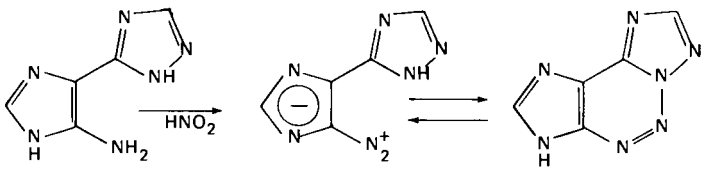
(165)

*O*-acetyl-β-D-ribofuranoside [313]. The thiol (168) could be of considerable biological interest because it is the 2-aza analogue of the clinically useful anticancer drug 6-mercaptopurine.



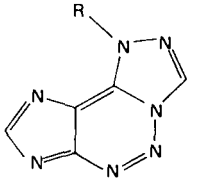
(166) (167) (168)

Entry into the tricyclic imidazotriazine field can be achieved by diazotisation of 3-(5-aminoimidazol-4-yl)-*s*-triazole (169). A mixture of the diazoimidazole (170) (the negative charge may also be delocalised in the triazole ring) and imidazo[4,5-*e*]-*s*-triazolo[1,5-*c*][1,2,3]triazine (171) is obtained [314]. The diazo derivative, in the form of its sodium salt, exists entirely as the anion of the cyclic species, but in TFA only the (protonated) diazo form can be detected. The tricyclic partner is stable in 2-methoxyethanol and DMSO-$d_6$, but addition of TFA in increments to the DMSO-$d_6$ solution shows a corresponding liberation of the diazo derivative.

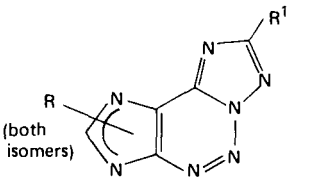


(169) (170) (171)

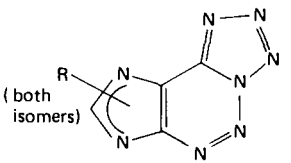
Other related derivatives which have been employed to examine the diazo-$\beta$-azomethine–1,2,3-triazine equilibrium include the benzylimidazotriazolotriazine (172), where the nature of the substitution necessitates ring-fusion at the unfavoured $N(4)$ position of the triazole, the $N(1)$ fused triazoles (173) and the imidazotetrazolotriazines (174) [315].



(172)
R = Benzyl

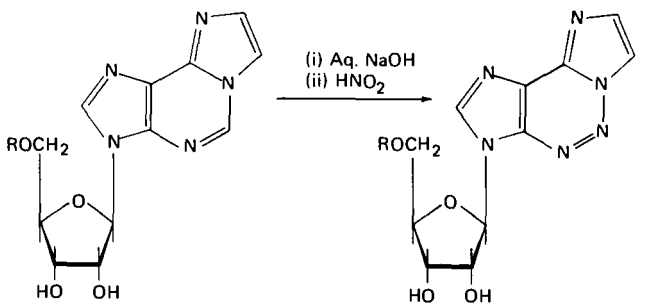



(173)
R = Benzyl
$R^1$ = H, OH




(174) R = H, benzyl

Synthesis of some biologically-interesting nucleosides starts with $1,N^6$-ethenopurines (175a–e): The pyrimidine rings are successively ring-opened and deformylated with aqueous sodium hydroxide, and cyclised



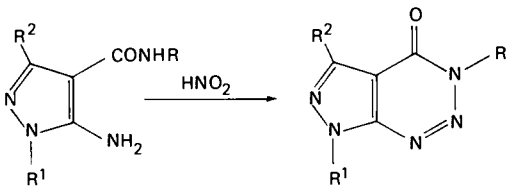

(175)
(a) R = H
(b) R = $PO_3^{-1}$
(c) R = $P_2O_5^{-2}$
(d) R = $P_3O_9^{-3}$
(e) R = 3′, 5′, –cyclic $PO_2^-$

(176)

with sodium nitrite in acetic acid [316]. One such product, 2-aza-1,$N^6$-ethenoadenosine (176a), is selectively toxic towards rat mammary tumour (AC-33) although inactive against HeLa cells and Glioma 26 cells. All the 2-azaethenoadenosine derivatives (176a–e) are highly fluorescent and can be detected in concentrations of the order of $10^{-7}$ M.

## PYRAZOLO- AND TRIAZOLO[1,2,3]TRIAZINES

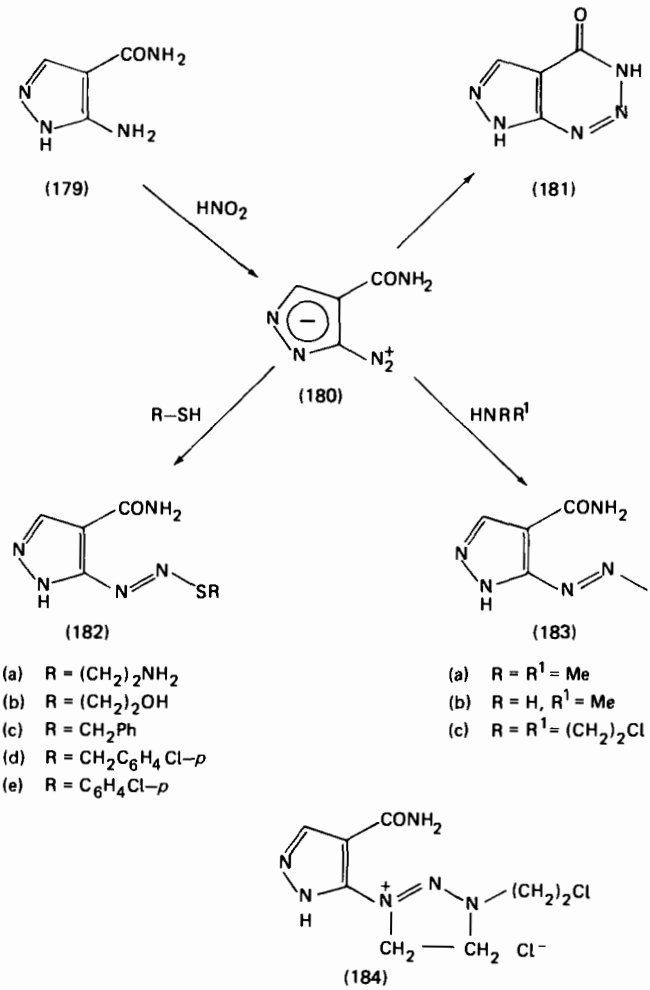
The first synthesis of the pyrazolo[3,4-*d*][1,2,3]triazine ring-system was achieved by Justoni and Fusco who cyclised substituted aminopyrazoles (177a and b) to the pyrazolotriazinones (178a and b) with acidified nitrite [317]. A recent study of the cyclisation of 1-methyl-5-aminopyrazole-4-carboxamide (177c) has revealed that either the pyrazolotriazinone (178c) or 1-methyl-5-nitrosoaminopyrazole-4-carboxamide may be formed depending on the reaction conditions [318]. Stable diazopyrazole intermediates are not possible in these nitrosations.




(a) R = H, $R^1$ = $R^2$ = Ph
(b) R = Me, $R^1$ = $R^2$ = Ph
(c) R = $R^2$ = H, $R^1$ = Me
(d) R = $R^2$ = H, $R^1$ = Ph
(e) R = Me, $R^1$ = Ph, $R^2$ = H
(f) R = $(CH_2)_2NMe_2$, $R^1$ = Ph, $R^2$ = H
(g) R = H, $R^1$ = $C_6H_4Cl$–*p*, $R^2$ = H

Related derivatives (178d–g) are claimed to have valuable diuretic properties [319, 320]; compound (178e), although a weaker diuretic than theophylline, is seven times less toxic [320].

Diazotisation of 3-aminopyrazole-4-carboxamide (179) leads initially to the diazopyrazole (180) which cyclises on standing to 7*H*-pyrazolo-[3,4-*d*][1,2,3]triazin-4(3*H*)-one (181). The diazopyrazole couples with the Bratton–Marshall reagent whereas the cyclic triazinone does not [321].

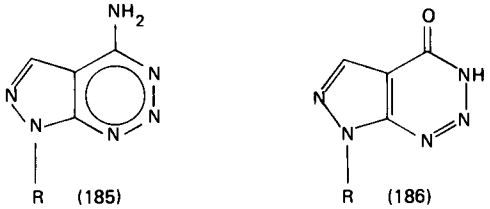



Both the diazopyrazole and the pyrazolotriazinone (a 2-aza analogue of allopurinol), are effective inhibitors of xanthine oxidase [275, 321]. As in the case of the corresponding imidazole derivatives, a common intermediate (probably the diazopyrazole) may be responsible.

The diazopyrazole couples with thiols to yield a series of (thioazo)pyrazole carboxamides (182a–e). In addition to their tumour-inhibitory and immunosuppressant activity, these compounds are active against gout and the Lesch–Nyham syndrome [322]. Interaction of the

diazopyrazole (180) with a range of dialkylamines affords triazenopyrazoles with chemical and biological properties similar to those of the corresponding triazenoimidazoles [271, 323, 324]. Initial evidence suggested that the antileukaemic pyrazole analogue of DIC (183a) was stable in light and heat [323] but this report has been modified [324]. The light-catalysed transformation product has not been identified but is presumably the pyrazolotriazinone (181). The monomethyltriazenopyrazole (183b) decomposes in the dark to 3-aminopyrazole-4-carboxamide (179) in a process exactly parallel to the MIC→AIC transformation in the imidazole series [324] (see pp. 248–249). A further similarity is shown by the bis(2-chloroethyl)triazenopyrazole (183c) which cyclises in the dark to the triazolinium salt (184) [324].

4-Amino-7-β-D-ribofuranosylpyrazolo[3,4-*d*][1,2,3]triazine (185) may be prepared from the corresponding pyrazolo[3,4-*d*]pyrimidine riboside by the route previously described: *N*-oxide formation, *O*-benzylation of the *N*-oxide, ring-opening and deformylation, followed by nitrosative ring-closure [5]. It is only marginally active against H.Ep.-2 cells.



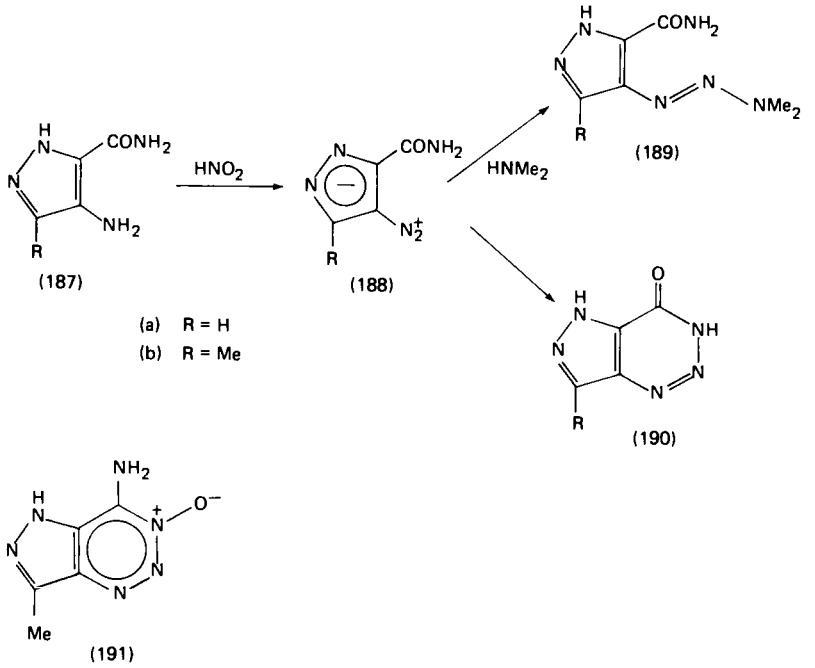

(185) (186)

R = β–D–ribofuranosyl

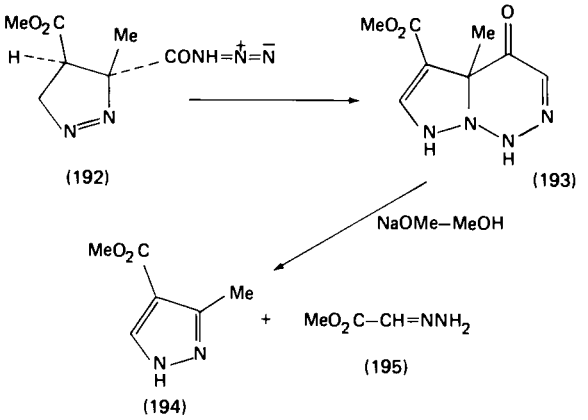
7-β-D-Ribofuranosylpyrazolo[3,4-*d*][1,2,3]triazin-4(3*H*)-one (186) may be likewise prepared indirectly from the corresponding pyrazolopyrimidinone, or by enzymatic deamination of riboside (185) with adenosine deaminase [257]. It is not cytotoxic.

Diazotisation of 4-aminopyrazole-3-carboxamides (187a and b) yields unstable diazo derivatives (188a and b) which couple with dimethylamine to afford dimethyltriazenopyrazoles, one of which (189a), differs from the antileukaemic agent (183a) only in the positions of the heteroatoms in the pyrazole ring [325]. Alternatively, intramolecular cyclisation of the diazopyrazole (188b) gives an entry to the pyrazolo[4,3-*d*][1,2,3]triazine system (190b) [326]. Similarly, diazotisation of 4-amino-3-methyl-5-carboxamidoxime affords the aminotriazine *N*-oxide (191).

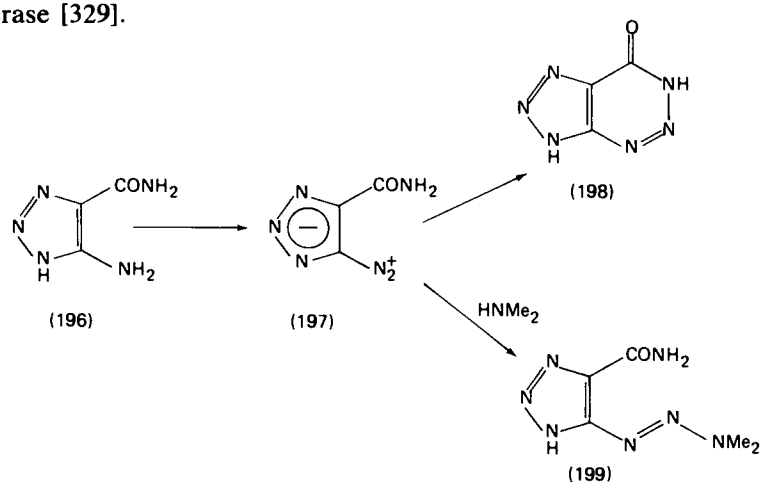
An entirely novel reduced pyrazolo[1,5-*c*][1,2,3]triazinone (193) has




been prepared by base treatment of the 3-diazoacetylpyrazoline (192). The triazinone is unstable and decomposes in sodium methoxide–methanol to yield methyl 3-methylpyrazole-4-carboxylate (194) and the hydrazone of methyl glyoxylate (195) [327].

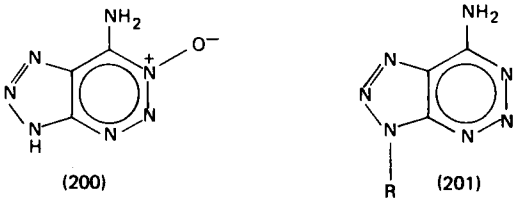


Relatively few examples of the *v*-triazolo[4,5-*d*][1,2,3]triazine ring-system are known. Nitrosation of 5-amino-*v*-triazole-4-carboxamide (196), as expected, gives first the diazotriazole (197) [260] which is more stable in 0.1 N hydrochloric acid (but less stable at pH 7) than the corresponding diazoimidazole [328]. The cyclised product, 2,8-diazahypoxanthine (198), is a more effective inhibitor of uricase than either 2-aza- or 8-azahypoxanthine but is inactive against milk xanthine oxidase and rat erythrocyte hypoxanthine-guanine phosphoribosyltransferase [329].




The diazotriazole couples with mono- and di-alkylamines to form tumour-inhibitory triazenes. 5-(3,3-Dimethyl-1-triazeno)-*v*-triazole-4-carboxamide (199) significantly increases the life-span of mice bearing L 1210 leukaemia, and 2,8-diazahypoxanthine shows borderline activity against sarcoma 180 [328]. Structure–activity relationships in a series of triazenotriazoles closely parallel those of the triazenoimidazoles [271, 283].

2,8-Diaza-adenine-1-oxide (200) and 2,8-diaza-adenosine (201) can be

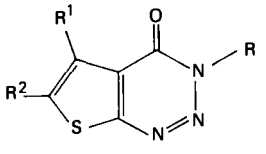



R = β–D–ribofuranosyl

prepared from 8-aza-adenine-1-oxide and 8-aza-adenosine respectively [5, 255] but do not have any outstanding biological properties.

## 1,2,3-TRIAZINES FUSED TO SULPHUR HETEROCYCLES

Examples of the three possible thieno-*d*-[1,2,3]triazine ring-systems are known, but the lack of progress in this area stems mainly from difficulties inherent in the synthesis of suitable precursors. The thieno[2,3-*d*][1,2,3]triazinones (202) are prepared by conventional diazotisation of 2-aminothiophen-3-carboxamides and are claimed to possess sedative, analgesic and anti-inflammatory activity [330].

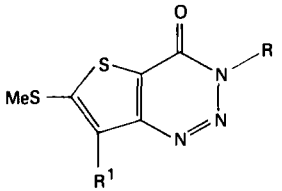


(202)

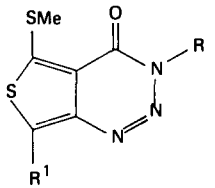
R = H, Me, $CH_2COPh$
$R^1R^2$ = H, Me, $(CH_2)_4$

Similar cyclisations afford derivatives of the thieno[3,2-*d*]- (203) and thieno[3,4-*d*][1,2,3]triazine (204) ring-systems[331], and the tricyclic triazinone (205) [332].

Similar difficulties attendant on the synthesis of precursors has restricted the availability of other hetero-fused 1,2,3-triazines. The

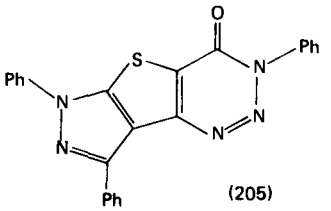


(203)



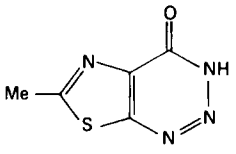
(204)

R = H, Me
$R^1$ = CN, $CO_2Me$, $CO_2Et$, Ac, PhCO, $CONH_2$, CONHMe

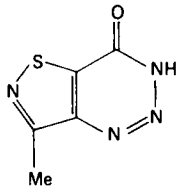


(205)

thiazolo[5,4-*d*][1,2,3]triazinone (206) [333], isothiazolo[4,5-*d*]- (207) [334] and isothiazolo[4,3-*d*][1,2,3]triazinones (208) [335] appear to be the only known representatives of these systems, and furo-, oxazolo-, and isoxazolotriazines are as yet unknown.




(206)

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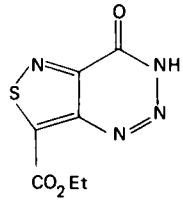


(208)

A major synthetic effort in this field would be valuable. A comparison could be made between the photolytic and thermal decompositions of sulphur- or oxygen-containing triazinones and their carbocyclic analogues (the 1,2,3-benzotriazinones)–such work could give a new insight into the mode of decomposition of these compounds (see pp. 220–221); furthermore, it could lead to novel heterocycles which would be attractive candidates for biological screening.

## CONCLUSION

The close parallel between the chemical properties of aryldiazonium compounds and 1,2,3-triazines fused to an aromatic carbocyclic or heterocyclic ring has been repeatedly stressed in this review. Although both structural types display a range of interesting biological properties, their often intriguing chemical features have not yet been reflected in *useful* medicinal activity. A medicinally desirable diazonium compound (if one were to be discovered) would test the skills of the most talented pharmaceutical formulator because of the inherent instability of this structural type: fused 1,2,3-triazines on the other hand are, in general, stable crystalline compounds readily amenable to manipulation yet lacking little in chemical versatility.

If it proved possible to obtain definitive evidence for covalent interactions between fused 1,2,3-triazines and biologically-important macromolecules, it would remove prospects for a biological role for these compounds from the realm of speculation to a firm scientific base. A suitable starting point for such studies might involve an examination of

the interactions of the bio-active azolo[1,2,3]triazines, and it is to be hoped that this article may stimulate increased efforts in this area.

Finally, it should be noted that only a small proportion of the many structurally diverse 1,2,3-triazines which have been prepared over the years have been examined for biological activity. Although interpretation of biological results is often difficult–most of the biological results recorded in this review are presumably authenticated, some are mere claims, and a few no doubt imaginary–in the author's view there is little merit in restricting interest in these compounds to their chemical and physical characteristics. It was one of the objectives of this review to make a case for a fuller biological evaluation of 1,2,3-triazines, and perhaps the next review on the subject will report on major advances in the field, and be more retrospective than prophetic in content.

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# 4 The Biological Uses and Importance of Glutaraldehyde

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## INTRODUCTION

The first report on glutaraldehyde was published by Harries and Frank [1] who synthesized the dialdehyde by boiling the ozonide of cyclopen-

tene with water to give several products, one of which was glutaraldehyde. It remained an obscure, little-known substance until research by leather chemists resulted in its trial as a tanning agent; subsequently, glutaraldehyde fulfilled a useful role within this industry [2]. The commercial availability of glutaraldehyde led to further uses, e.g. in electron microscopy, where it is a superior fixative possessing advantages over its predecessors [3], as a cross-linking agent for proteins and enzymes, and as a chemosterilizer for hospital equipment [4]. The uses of glutaraldehyde are described more fully later.

The modern industrial production of the aldehyde involves a two-step synthesis from an interaction of acrolein with vinyl ethyl ether to produce an ethoxy dihydropyran which is then hydrolysed with water to form glutaraldehyde and ethanol [5] (*Figure 4.1.*).

Today, glutaraldehyde is used in three major areas: leather tanning, sterilization and disinfection, and tissue fixation for electron microscopy. This review is concerned mainly with the latter two of these subjects.

## CHEMICAL ASPECTS

Glutaraldehyde is a saturated 5-carbon dialdehyde with an empirical formula of $C_5H_8O_2$ and a molecular weight of 100.12. Harries and Frank [1] described it as a colourless, mobile liquid, although in commercial samples it often has an amber tint and a distinctive odour similar to 'spoiled fruit' [6]. It is usually supplied as a 2, 25 or 50% solution of acidic pH. Under suitable conditions, its two aldehyde groups undergo most of the typical aldehyde reactions; these groups may react singly or together

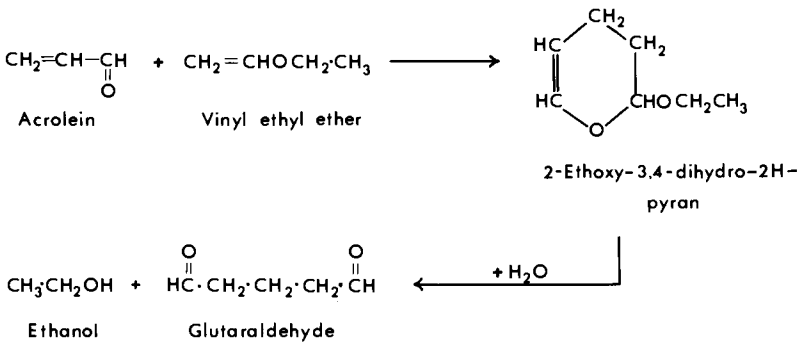



*Figure 4.1. Industrial production of glutaraldehyde*

to form bisulphite complexes, oximes, cyanohydrins, acetals and hydrazones as do other aldehydes [7]. The dialdehyde also reacts with proteins [8] by a cross-linking reaction, which is primarily between $-NH_2$ groups and which is influenced by time, pH and temperature [9].

Polymerization of the glutaraldehyde molecule is known to occur and consequently the chemical structure of the dialdehyde is by no means clear. Differing proposals have been put forward as to the form and nature of this polymerization. Rubbo, Gardner and Webb [10] proposed that the dialdehyde existed as a monomer and that an equilibrium was established between the open chain molecule (approx. 25%; *Figure 4.2a*) and the hydrated ring structure (*Figure 4.2b*). Hardy, Nicholls and Rydon [11] also postulated that the monomer existed as a mixture of hydrated forms in aqueous solution. Aso and Aito [12, 13] polymerized glutaraldehyde using cationic catalysts; the final product contained few residual aldehyde groups, suggesting that ring formation occurred during the propagation process, which is by an intramolecular mechanism. They also showed that polymerization, in a similar manner, occurred spontaneously in aqueous solutions kept at room temperature in the absence of a catalyst, to give a soluble pentamer or tetramer containing about 1 free aldehyde group per molecule. Similarly, Moyer and Grev [14], using an aluminium catalyst, described the production of a very high molecular weight polyglutaraldehyde which was soluble in water but thermally unstable; this had the same unit structure (*Figure 4.3*) as that proposed by Aso and Aito [12, 13]. Yokata, Suzuki and Ishii [15] also showed that glutaraldehyde existed as a trimer above 0°C.

Another structure has recently been advocated as a result of the nuclear magnetic resonance (NMR) spectroscopy study made by Richards and Knowles [16], who found that commercial glutaraldehyde was largely polymeric and contained significant amounts of $\alpha,\beta$-

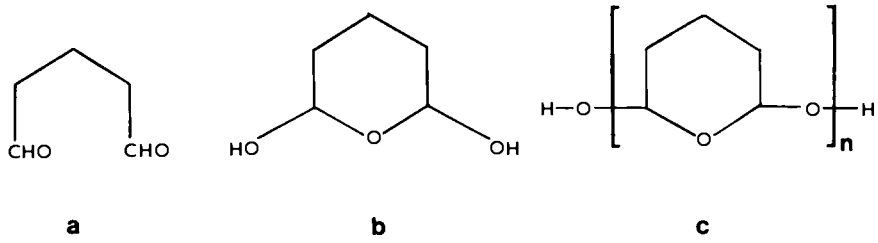



*Figure 4.2. (a) Free glutaraldehyde. (b) Cyclic hemiacetal of its hydrate (i.e. hydrated ring structure). (c) Oligomer*

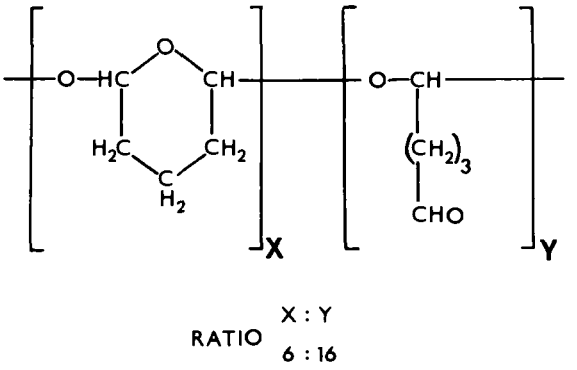



*Figure 4.3. Polymer of glutaraldehyde proposed by Aso and Aito [12, 13]*

unsaturated aldehydes resulting from the loss of water by aldol condensation. No one specific polymeric form was indicated, but rather a mixture of dimer, cyclic dimer, trimer or bicyclic trimer and higher polymeric species.

Fein, Harris, Naghski and Filachione [2] have shown that polymerization increases with a rise in pH and that above pH 9 there is an extensive loss of aldehyde groups.

Korn, Feairheller and Filachione [17] proposed that aqueous solutions of glutaraldehyde consist of free glutaraldehyde (*Figure 4.2a*), the cyclic hemiacetal of its hydrate (*Figure 4.2b*), and oligomers of this (*Figure 4.2c*) in equilibrium. They also point out that glutaraldehyde polymerizes in a different manner under alkaline conditions, and does not then react well with proteins. Others [18–20] have proposed that the monomeric glutaraldehyde is the active species in terms of cross-linking with proteins and that the facility of 'polymers' to revert to the active monomer (*Figure 4.4a*) depends upon pH, i.e. the type of polymers in solution. They consider that the polymers in the alkaline range (*Figure 4.4c*) cannot revert to the monomer and have a short life as sterilising agents (see later) because time and temperature tend to produce a more irreversible polymer. In contrast, polymers in the neutral and acid range (*Figure 4.4e*) are considered to revert easily to the monomer under the influence of heating or ultrasonic radiation.

Thin layer chromatography of the 2,4-dinitrophenylhydrazones of glutaraldehyde on zinc carbonate has been reported by Beyer and Karge [21]. A pink-purple colour revealed the derivatives when the plate was wet with pyridine.

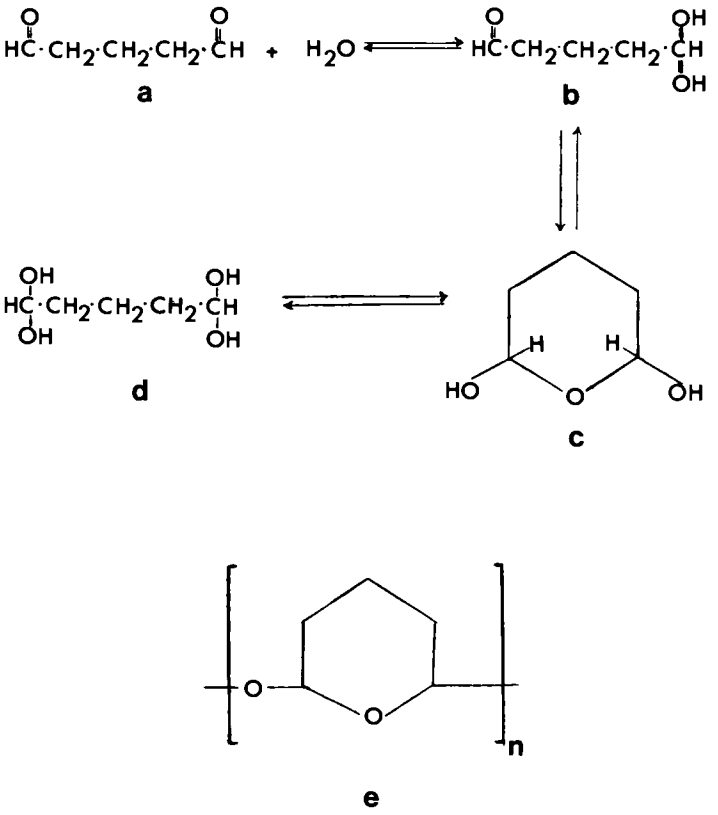



*Figure 4.4. Glutaraldehyde (a) Open chain molecule. (b), (c), (d), Formation of several more stable 'polymers' (hydrated) in aqueous alkaline solutions. (e) A polymer with an acetal-like structure, in neutral and acid ranges*

## PURITY OF GLUTARALDEHYDE

Morphologists have been interested in the purity of glutaraldehyde used for fixation, as the quality of this process appears to depend on the purity of the aldehyde [22, 23]. The criterion most used is the ratio of absorbance at 235:280 nm. Various methods have been introduced to remove the 235 nm absorbing material [22–24]. Gillett and Gull [25] have claimed that storage temperature is the most important factor preventing polymerization and that light and inert atmosphere play little part. Rasmussen and Albrechtsen [26] more recently have shown that the rate of polymeriza-

tion depends exponentially on temperature. Hydrogen ion concentration is also important, there being a sharp rise in polymerization on either side of neutrality [26] (see *Figure 4.4e*, also). Hesse [26a] investigated the change in absorbance over the first hour, and this was found to rise at a rate depending on the purity and concentration of the glutaraldehyde. Goff and Oster [27] found that during fixation of tissues, material absorbing at 235 nm increased in amount.

## BIOLOGICAL USES OF GLUTARALDEHYDE

### GENERAL AND MISCELLANEOUS ASPECTS OF FIXATION

The role of glutaraldehyde as a fixative has been reviewed previously [24]. Recently, Dorsche and Wolter [28] have extended earlier work [6] on the use of glutaraldehyde as a general fixative. They showed that only lipids are lost. All the histological staining methods were successful. When histochemical techniques for proteins were tried, only the ninhydrin–Schiff and tryptophan methods worked. They also reported that some heavy metals were identified.

Fitzharris, Bloodgood and McIntosh [29] produced some interesting evidence to show that fixation is not instantaneous. They found that the microtubular axostyle of the termite gut protozoan *Pyrsonympha* made a sensitive bio-assay for analyzing the rate of fixation using cine recording. They showed a gradual decrease in the velocity of wave propagation without change in wave characteristics over a period of several seconds. They stressed the importance of this in the interpretation of ultrastructure as, for example, chromosomes can move 0.5 $\mu$m in 10–30 seconds.

Similar information on the time taken for fixation to occur was deduced by Hubbard and Laskowski [30] who assessed spontaneous transmitter release and sensitivity to acetylcholine in rat phrenic nerve-diaphragm preparations during glutaraldehyde fixation. The miniature end-plate potential and frequency and amplitude declined significantly within 45 seconds of exposure. This time is longer than that for *Pyrsonympha*, but here, whole tissues are being used rather than protozoa and the cessation of function also depends on the rate of penetration of glutaraldehyde, which is slow [24].

Related to these phenomena of loss of organised function with fixation, is some recent work by Penttila, Kalimo and Trump [31], who used Erlich ascites tumour cells. They thoroughly investigated a number of variables

including time of fixation, fixative concentration, osmolarity of buffer and fixative and their sum osmolarity of the post fixative buffer, and the time spent in it. They showed there was a rapid loss of potassium, ATP and exchangeable magnesium, and increased uptake of vital dye and electrical conductivity. This is due to rapid change in membrane permeability following glutaraldehyde and osmium tetroxide fixation, probably due to the interaction of the protein with these fixatives. Previous literature in this area varies in its interpretation [24, 32], but these results [31] seem reasonable when considered from first principles. The greater mechanical, osmotic and thermal stability after fixation was accompanied by an increase in the negative charge on the cell surface [33]. Yip and Auersperg [34] have found that vital dyes penetrate more slowly into glutaraldehyde-fixed cells when they are stored in the aldehyde rather than in buffer.

Lee [35] has noted that cells vary in their resistance to collagenase (EC 3.4.24.3) and trypsin (EC 3.4.21.4) following fixation with 1% glutaraldehyde. He used this observation to make 95% pure preparations of parietal of rabbit stomach cells by gradient centrifugation. This general technique is applicable in other areas.

There are various reports of unsatisfactory fixation of plant and other specimens by glutaraldehyde [24, 32]. Borgens and de Nollin [36] found this when they tried conventional technique for fixing *Candida albicans*, and it is due to slow penetration beyond the cell wall and plasmalemma. They found that when the cells were harvested, pelleted and fixed in 2% glutaraldehyde, 7 $\mu$m sections cut and then post-osmicated, they achieved satisfactory morphological preservation.

The chemical basis for the argentaffin reaction applied to the glutaraldehyde-fixed tissue has been studied [37]. In vitro chemical tests showed that a precipitate which was not chemically homogeneous was formed between glutaraldehyde and 5-hydroxytryptamine. We have made similar reports previously with biogenic amines [38]. Tetrahydro-$\beta$-carboline was formed, and this precipitates ammoniacal silver at the site of storage of the 5-hydroxytryptamine [37].

There is a report from the patent literature [39] that glutaraldehyde included in chewing gum has anti-caries properties.

Another recently introduced method by Futaesku [40] utilized 4% tannic acid with 2.5% glutaraldehyde, again producing better morphological results. The author suggested that complexes may be formed: protein–tannic acid–metal (osmium or uranium).

The problem of artefacts is a serious one for electron microscopists [24, 41]. Higgins and Daneo-Moore [42] have recently reported on the

influence of fixation on the appearance of mesosomes in *Streptococcus faecalis*. Following osmium tetroxide or glutaraldehyde fixation, mesosomes were present in up to 80% of sections or fractures in freeze-etching. After freeze-fracture alone, then the rate of occurrence fell to 8%, rising to 50% when glycerol was used. These results pose interesting questions.

## COMPARISONS OF FIXATIVES AND PREPARATIVE TECHNIQUES

Recently a number of papers have been published which compared a single tissue prepared by different fixatives. Busson-Mabillot [43, 44] has investigated the oocyte of a teleost. Proteins were preserved best with glutaraldehyde, whereas lipids and glycoproteins were best fixed by osmium tetroxide. Simson, Spicer and Hall [45] found similarly in secretory granules in rat salivary glands and also that a mercuric chloride–potassium dichromate mixture preserved glycolipids well at the ultrastructural level. This is of interest, as the fixative effects of mercuric chloride have not been found to be good for fine structure [32].

Investigation of the effects of a number of fixatives on plant cells from the root tip of *Phaseolus vulgaris* both at the light- and electron microscopic levels, including glutaraldehyde, osmium tetroxide, formaldehyde, acrolein, potassium dichromate, Clarke's fluid and chromic acid, acetic and water showed that glutaraldehyde was an excellent general fixative [46]. However, all the agents produced some artefacts and the authors warned against these.

There have appeared recently, a number of papers by Weakley, comparing the effects of various preparative techniques on the fine structure of hamster ovarian tissue [47, 48]. The nuclear morphology was found to be independent of the type of fixative. EM grade glutaraldehydes from 3 suppliers were compared [48] for the pH of stock solution, the 235/280 nm UV absorbance ratio, milliosmolarity and quality fixation of ovarian tissue. The most detrimental factor appeared to be low pH, although a low 235/280 nm ratio (0.18–0.67) also gave poor fixation. Variations in milliosmolarity appeared to have little effect. The best results were obtained with stock solutions with pH greater than 4 and a 235/280 nm ratio of 1–2.

Iqbal and Weakley [49] systematically investigated the effects of fixative concentration and buffer osmolarity on the fixation of hamster oocytes and their granulosa cells. The glutaraldehyde concentration had

little effect on ultrastructure between 2–4%. At 1% the fixation is erratic. The buffer ions probably precede the fixative and are of more importance, although the practice of adding sucrose to the fixative is probably irrelevant as far as glutaraldehyde solutions are concerned. The optimum osmolarity appears to be between 195 and 225 mOsm, and below 95–115 mOsm swelling and leaching occur. The reason for better fixation by slightly hyperosmolar solutions is not known for certain, although various hypotheses are discussed. Bohman and Maunsbach [50] have previously shown that the colloid osmotic pressure can alter the tissue volume. However, more recently, Penttilla, Kalimo and Trump [31] have shown that total fixative osmolarity has little effect after glutaraldehyde fixation, but marked effect after osmium tetroxide. These authors also point out that certain features appear in the ovary, regardless of the preparative technique. These include dark cells, which occur in various situations. They have given rise to much discussion and it has been claimed on one hand that they are fixation artefacts [51] and on the other, that they represent effete, atrophic or apoptotic cells [52].

Diers and Schieren [53] have investigated the effects of fixation and embedding medium on the size of chloroplasts in *Elodea* by various techniques. They found that osmium tetroxide caused swelling which was preserved by Araldite and lost with methacrylate. Glutaraldehyde produced a shrinkage, on the other hand. However, when the *Elodea* was post-fixed in osmium tetroxide, the dimensions became about the same as in the living.

## LOSS OF MATERIALS

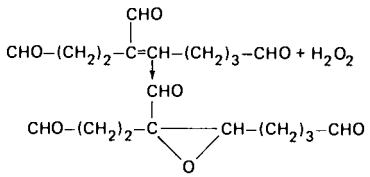
The loss of materials from tissues during fixation is a problem at the ultrastructural level. There is relatively little information available in this area [24, 32]. The loss of lipids after glutaraldehyde fixation is discussed elsewhere in this paper. Simson, Spicer and Hall [45] have shown in a study in which they compared the effects of a number of fixatives on this tissue, that different materials are lost from rat salivary gland secretory granules, including glycolipids. The loss of bile acids and their glycine and taurine conjugates from the liver during preparation of tissue for electron microscopy fixed with glutaraldehyde osmium tetroxide was about 30%, mostly due to the acetone dehydration [54]. Previously, Coupland and Hopwood [38] showed that 37% of the adrenalin from rat adrenals were lost into the glutaraldehyde fixative and a further 50% of the control value into the dehydrating fluids. Van Harreveld and Fifkova [55] have shown

that glutamate is released from the retina during its fixation with glutaraldehyde. The glutamate reacts with the cell membrane and causes a contraction of the extracellular space. Dorsche and Wolter [28] made a study of glutaraldehyde as a general fixative for histology and commented that the only substances apparently lost were the lipids.

## RECENTLY INTRODUCED GLUTARALDEHYDE-CONTAINING FIXATIVES

There is a general hope in histological procedures, that the short-comings of one fixative may be compensated by adding another. There are various examples in the literature of fixatives for electron microscopy [24, 32].

Recently several modifications of glutaraldehyde fixation have been introduced by Peracchia and Mittler [56, 57]. They found that the addition of hydrogen peroxide to the fixative improved the final tissue morphology, compared with the conventional technique. Fine detail in tissues is less distorted in difficult plant and animal specimens. The concentration of the hydrogen peroxide appears to be critical. The originators of the method investigated the chemical nature of the reaction products by NMR spectroscopy and found that epoxides (for example, (1)) were primarily formed, probably from $\alpha,\beta$-unsaturated aldehydes, the products of aldol condensation always present in commercial glutaraldehyde. The epoxides react readily with amino, imino, hydroxyl and mercapto groups.



(1)

Glutaraldehyde–hydrogen peroxide fixation has been used in cytochemical studies by Goldfischer, Essner and Schiller [58]. They found that preservation of thiamine pyrophosphatase (thiaminephosphate pyrophosphorylase, EC 2.5.1.3) and nucleosidediphosphatase (EC 3.6.1.6) was better than after conventional glutaraldehyde fixation. They believed that penetration was more rapid.

Peracchia and Mittler [57] also investigated the effect of temperature on tissue fixation with glutaraldehyde. Tissues were fixed initially at room temperature and subsequently at 45°C for two hours. Again, the resulting morphology was better than that produced by conventional means. The authors suggested the mechanism was the polymerization of glutaraldehyde. The different length polymers then would be able to cross-link reactive groups of different substances. However, it has been shown that increasing the temperature increases the amount of free aldehyde in solution [17, 59].

## GLUTARALDEHYDE AS AN EMBEDDING MEDIUM

Pease [60] initiated work in the use of glutaraldehyde to embed specimens. The early work produced a gel which gave marginal support only. The methodology was modified successfully and urea added by analogy with urea formaldehyde. Subsequently, Yarom, Peters and Hall [61] have used this technique to study intracellular ionic elements of heart and skeletal muscle. Theoretically, the glutaraldehyde urea method should cause less disturbance of intracellular electrolytes. The technique was found preferable to Epon for histochemistry and electron X-ray micro analysis, both analytically and visually.

## BIOCHEMICAL USES

Deamer, Utsumi and Packer [62] showed that glutaraldehyde trapped the configurational state of mitochondria, and Packer and Greville [63] that at low concentrations it stimulated their oxygen uptake, possibly acting as a substrate [64] for an aldehyde oxidase. Preservation of enzyme activity, particularly electron transport, occurs in treated mitochondria [65]. By controlling the degree of cross-linking produced by glutaraldehyde, it is possible to achieve fixation of ultrastructure whilst maintaining electron transport and other functions, e.g. in chloroplasts [66–68].

This feature of structural preservation with persistence of enzyme activity occurs in several reports. Papain (EC 3.4.22.2) insolubilized with 2% glutaraldehyde retains significant esterolytic and proteolytic activity [69] and subtilisin treated with the aldehyde also retains considerable activity [70]. However, Wang and Tu [70a] found that incubation of glycogen phosphorylase *b* (EC 2.4.1.1) with glutaraldehyde resulted in inactivation of the enzyme. Nevertheless in certain cases, a fraction of the glutaraldehyde-treated enzyme is highly resistant to denaturation and

other protein degradative techniques without becoming inactive [71, 72] so that glutaraldehyde may find widespread use in the field of enzyme stabilization.

Not surprisingly, glutaraldehyde is now being used in immunology [73, 74]. By utilizing its cross-linking ability, enzymes may be attached to proteins which retain their immunological activity and which are readily identifiable by the attached enzyme activity. This conjugation of different proteins by glutaraldehyde is not, however, limited to immunology: reports by Sachs and Winn [75] and others [76] indicate that it is being widely used for attaching enzymes as markers to proteins.

## MICROBIOLOGICAL ACTIVITIES AND USES

The term 'chemosterilizer' as used here, denotes a substance which destroys all forms of microbial life including bacteria and their spores, fungi and viruses [77, 78]. During a search for an efficient substitute for formaldehyde, Pepper and Lieberman [79] tested a group of saturated dialdehydes for antibacterial, and especially sporicidal, activity. The findings that certain of these compounds in alkaline isopropanol were sporicidal prompted further studies in which alkaline glutaraldehyde was found to be a superior sporicidal agent to both formaldehyde and glyoxal [80]. Sodium bicarbonate, 0.3% w/v, was used for alkalinization: without its addition there was negligible sporicidal activity [81].

Glutaraldehyde was now established as an effective sporicidal agent, and towards the end of 1963 a 2% solution was marketed ('Cidex') by Ethicon, Inc., this being 'activated' with 0.3% w/v sodium bicarbonate before use to give a chemosterilizer. Alkaline glutaraldehyde has been shown to be rapidly lethal to bacteria, including bacterial spores [4, 82–84; cf. 85, 86], various types of viruses [87–89] and both the mycelial and spore forms of fungi [10, 90]. Some doubt still remains as to whether glutaraldehyde is tuberculocidal, and it has been claimed that in this context it is apparently less effective than either formaldehyde or iodine [10, 91]. However, Spaulding [92] has classified antimicrobial agents into three groups:

(a) 1st level (high): killing spores, tubercle bacilli and lipid- and non-lipid viruses;

(b) 2nd level (intermediate): killing tubercle bacilli and lipid- and non-lipid viruses;

(c) 3rd level (low): killing vegetative bacteria and lipid viruses only.

Spaulding considers that glutaraldehyde belongs to the first group, i.e.

it is an excellent chemosterilizer. However, it has been suggested [93] that glutaraldehyde has only a low order of activity against *Pseudomonas aeruginosa.*

These overall findings have resulted in the introduction of glutaraldehyde as a chemosterilizer for certain types of medical equipment. Rittenburg and Hench [93a] concluded that haemostats, cystoscopes, food containers and anaesthetic equipment (in some cases contaminated with blood or pathogenic bacteria) could be effectively sterilized by immersion for short periods (5 min) in cold glutaraldehyde solutions. Because of its rapid lethal activity and lack of deleterious effects, glutaraldehyde has been further recommended by other authors [94–98] for these purposes. In short, it is a particularly useful agent in the sterilization of medical equipment which cannot be sterilized by more physical methods [99]. Its use as a preservative in cosmetics has been studied [100].

The use of buffered glutaraldehyde as a chemosterilizer for hospital equipment is now widespread. The main advantages claimed for its use as a chemosterilizer are:

(i) broad spectrum of activity;

(ii) rapid microbicidal action;

(iii) non-corrosive to metals, rubber, lenses and most materials;

(iv) mild odour, non-irritating (see section on toxicology also) and easy to use.

Nevertheless, some additional comments must be made concerning its antimicrobial activity. Organic matter is considered to have no effect on the activity of glutaraldehyde, although the interaction of the dialdehyde with amino groups in proteins suggests that this is a rather unusual finding. It might at present, and in the absence of further information on this point, be wise to reserve judgement. Secondly, dried spores are considerably more resistant to chemical disinfectants than are spores in suspension, and it would appear that glutaraldehyde is no exception in this context. The use of the AOAC test with dried spores of *B.subtilis* has shown that 2% alkaline glutaraldehyde may require up to 10 h to achieve sterilization at 20°C [10].

In the laboratory, it is customary to use aqueous suspensions of spores, and 2% alkaline glutaraldehyde is sporicidal at 20°C [83, 101] and markedly so at 37°C [83, 84]. At the latter temperature, 2% acid glutaraldehyde is also a quite effective sporicide, and at temperatures above 40°C differences in effect between alkaline and acid forms tend to disappear [84, 102].

Some comments must also be made as to the methods available for estimating glutaraldehyde activity. Ross [99], by analogy with studies on formaldehyde [103], used sodium bisulphite as an inactivating agent to prevent the carry-over of inhibitory glutaraldehyde levels into the recovery agar. However, sodium bisulphate has been found to increase the apparent lethal effect of glutaraldehyde against *E.coli* [104] and other bacteria [91]; this was, in fact, caused by the inhibitory action of the bisulphite itself. The use of sodium bisulphite as an inactivating agent for aldehydes has subsequently been criticised [86, 105, 106]. Kelsey, MacKinnon and Maurer [86] have recently proposed that in carrying out viable counts with glutaraldehyde-treated spores (a) serial dilution should be in nutrient broth or serum, (b) incubation of plates should be at 32°C rather than at 37°C, (c) the period of incubation should be at least 7 days. Thomas and Russell [107], however, have found that simple dilution in water, followed by plating in nutrient agar for 48 h is perfectly adequate, and that the enrichment of agar with serum, yeast extract or glucose does not give rise to increased survivor levels.

Recently, a glutaraldehyde product with increased stability and activity has been described [18, 20, 108]. Glutaraldehyde solutions are stable at acid pH values for at least 2 years [4], but under alkaline conditions the shelf life is only 2 weeks [82]; if, however, the temperature is kept at 4°C, then the period for storage of alkaline solutions is greater prolonged [84]. As already pointed out, glutaraldehyde is considerably less microbicidal at acid than at alkaline pH. However, at high temperatures, e.g. 70°C there is no significant difference in activity [102]; furthermore, ultrasonic treatment will increase the sporifical activity of 2% alkaline glutaraldehyde at 25°C [102], and a potentiated glutaraldehyde at acid pH is claimed to be as effective a sporicidal agent as alkaline glutaraldehyde [108], the potentiation being achieved by the addition of a specified non-ionic agent [109–111]. This could well be an important development in chemosterilization and disinfection.

A comparison of the bactericidal and sporicidal effects of acid, acid potentiated and alkaline glutaraldehyde is given in *Table 4.1.*

## TOXICOLOGY

Studies on the toxicity of glutaraldehyde have been designed mainly to determine possible hazards in its use as a chemosterilizer. Stonehill, Krop and Borick [4] considered that the compound was slightly to moderately toxic. A 2% solution is slightly irritant to the skin and severely irritant to

Table 4.1. SPORICIDAL AND BACTERICIDAL ACTIVITY OF GLUTARALDEHYDE

| | | *Bactericidal activity*[a] | | *Sporicidal activity*[b] | | | |
|---|---|---|---|---|---|---|---|
| *Form of glutaraldehyde* | *Approximate pH value* | *Gram positive* | *Gram negative* | *20–25°C* | *37°C* | *55°C* | *70°C* |
| Acid | 4–5 | Low | Low | Low | Increased[c] | V. high | V. high |
| Alkaline | 8 | High | High | Reasonable. | High | V. high | V. high |
| Potentiated | 5.5 | High | High | Reasonable. | | V. high | |

[a] Use of low dialdehyde concentrations (0.01–0.02%).
[b] Use of 2% dialdehyde concentrations.
[c] Activity of acid glutaraldehyde increases with temperature, and at 37°C its activity is approaching that of alkaline glutaraldehyde.

the eye but in both cases considerably less so than formaldehyde; however, contact with the skin should be avoided.

The $LD_{50}$ value for mice is 15 mg/kg weight [4]. Inhalation is severely toxic only with high ($>4\%$) aldehyde concentrations allowed to evaporate in a closed system. Stonehill, Krop and Borick [4] paid special attention to its action upon the urinary and respiratory tracts in connection with its use in sterilizing the relevant instruments, but it was concluded that residual amounts of glutaraldehyde after a sterilizing procedure were non-irritant.

Skin sensitization in hospital theatre staff who had handled instruments disinfected in glutaraldehyde has been reported with discolouration and hardening of the skin and subsequent development of a rash [112]. In contrast, however, a 10% glutaraldehyde solution has been employed to prevent hyperhidrosis (excessive sweating) of the feet with no cases of irritation [113]. Glutaraldehyde may, in fact, only be irritant if the epidermal barrier is not intact [114]. Gordon and Maibach [115] propose that the anhidrosis (deficient sweat secretion) caused by the aldehyde is due to abnormal keratinization and plugging of sweat duct orifices.

## USE IN LEATHER TANNING

The tanning properties of aldehydes have been the subject of numerous investigations, particularly in comparison to formaldehyde [116]. The introduction of glutaraldehyde gave new impetus to research in this field [117], thereby establishing the versatile tanning properties of this substance [118]. It was observed [118] that as the pH increased so the amount

of glutaraldehyde bound by the collagen and its rate of fixation increased, although tanning with the aldehyde could be achieved over a wide pH range, e.g. at pH 2, 5% of the total glutaraldehyde was bound in the first 30 min and 50% in 24 h, whereas 80% was bound initially at pH 8 [2, 119]. Glutaraldehyde is bound irreversibly to the collagen molecule [120] and severe acid hydrolysis is necessary to release it by the breaking of peptide bonds within the collagen rather than the actual glutaraldehyde binding site.

Between 4 and 5 molecules of glutaraldehyde are taken up for every lysine residue [8, 121]. Not all of this is firmly bound, however, because extraction with dioxan, which dissolves polymers of glutaraldehyde [12, 13] removes appreciable amounts of aldehyde material.

## MECHANISM OF BIOLOGICAL ACTION

### ACTIVITY OF FORMALDEHYDE

Glutaraldehyde is frequently compared with formaldehyde in its mechanism of attacking proteins and killing micro-organisms. Formaldehyde, in liquid and gaseous forms, has itself been used as an antimicrobial agent for almost a century [122]. The early interest in formaldehyde resulted from its importance in tanning leather [116], in the preparation of vaccines [123, 124] and in the preservation of tissues.

Formaldehyde displays many typical chemical reactions, combining with:

(i) amines to give methylolamines;
(ii) carboxylic acids to give esters of methylene glycols;
(iii) phenols to produce methylphenols;
(iv) sulphides to give thiomethylene glycols.

Fraenkel-Conrat, Cooper and Olcott [125] showed that formaldehyde interacted with protein by attaching itself to the primary amide as well as the amino group, although phenolic moieties bound little of the chemical. Subsequently, formaldehyde was found to give an intermolecular cross-linkage of protein amino groups with phenolic or indole residues [126, 127]. Loveless [128] postulated that the aldehyde was a mutagenic agent acting by the formation of organic peroxides from hydrogen peroxide, whereas an alkylating action with carboxyl, hydroxyl and sulphydryl groups has also been proposed [129].

In addition to reacting with many terminal groups in viral proteins,

formaldehyde can also react extensively with nucleic acids [130], e.g. it appears to react with the nucleic acid of tobacco mosaic virus in 2 steps: an initial loose binding, followed by a firm attachment after extensive reaction [130]. Formaldehyde reacts with the amino groups of bases and is much less reactive with DNA than with RNA. The second stable product of RNA and formaldehyde results from the formation of methylene bridges with adenosine [130, 131]. Sublethal concentrations of formaldehyde inhibit the synthesis of cytoplasmic and nuclear material in bacteria [132–134] but subsequently the aldehyde is metabolized to carbon dioxide, and cellular growth resumes [135].

Formaldehyde prevents the dissociation into sub-particles of reticulocyte [136] and bacterial [137] ribosomes, although it is likely that the reaction here is between formaldehyde and ribosomal protein, thereby leading to the formation of cross-links between the protein sub-units.

## INTERACTION OF GLUTARALDEHYDE WITH PROTEINS AND ENZYMES

The principal factor emerging from studies by Habeeb [138] and Habeeb and Hiramoto [139] is that glutaraldehyde reacts with various enzymes but does not sterically alter them to lose all activity. The main reactions occurred between glutaraldehyde and the $\alpha$-amino groups of amino acids, N-terminal groups of some peptides and the sulphydryl group of cysteine [139]. The phenolic and imidazole rings of tyrosine and histidine derivatives were particularly reactive. Some criticism of these latter findings has been made [17] that reactions with proteins involved principally the lysinyl residues in the relative amounts of 4 moles of glutaraldehyde to one of lysine.

The kinetics of protein–glutaraldehyde reactions have recently been studied [9]. Using 4% glutaraldehyde with concentrated protein solution, it was found that a yellow colour was produced which increased in intensity with time and that some proteins formed a gel. This gel formation depended upon the cross-linking of the protein which is again theoretically dependent upon lysine and other residual groups. By following the reaction spectrophotometrically, it was found that the rate of reaction was pH-dependent and increased considerably over the pH range 4 to 9, and that the reaction product was extremely stable and was not dissociated by dilution.

The expected reaction between an aldehyde and an amino group is the

formation of a Schiff's base [7]. However, Schiff's base formation is reversible, whereas the amino reaction with glutaraldehyde is not.

Since the earlier review [24] of the reactions between enzymes, proteins and glutaraldehyde, more papers have appeared. These confirm previous trends. One interesting paper in this field by Josephs, Eisenberg and Reisler [140] has investigated the polymerization of glutamate dehydrogenase (EC 1.4.1.2) with glutaraldehyde, comparing the physical state with enzyme activity. They found that the concentrations of protein and glutaraldehyde were most important in determining the nature of the product. With 4% glutaraldehyde, the concentration normally used for histology, an insoluble gel is formed, which contains over 90% of the protein originally present. These workers used lower protein and aldehyde concentrations to study the physical chemistry. They used gel filtration to separate polymers with molecular weights as high as $3 \times 10^6$. Electron micrographs of this material showed branch polymers of 10–20 units. The same authors also investigated the number of inter- and intra-molecular cross links formed and found the latter to be the more abundant. Kinetic parameters for activity of the glutarated glutamate dehydrogenase of different molecular weights show that activity of the fixed enzyme is independent of the degree of polymerization.

These findings highlight the problem of the morphologist. For good fixation with minimal loss of material, a high fixative concentration is optimal. If, on the other hand, he wishes to demonstrate enzyme activity histochemically, lower fixative concentrations must be used. For immunoelectron microscopy, where the active sites are on the surface of the antigens open to attack by the aldehyde, the problem is the more acute.

One area where fixation and denaturation may be put to some use is in the case of catalase (EC 1.11.1.6). Herzog and Fahimi [141] noted that glutaraldehyde apparently enhanced the peroxidative activity of catalase, when determined with DAB. This was confirmed chemically; other denaturing agents such as urea, heat and alkalinization had the same effect.

Payne [141a] has described conditions which yield covalently linked soluble protein oligomers after treatment with glutaraldehyde.

## INTERACTION WITH NUCLEIC ACIDS AND NUCLEI

The fixation of nuclei and nucleic acids is a subject which has been little investigated. In Baker's classic book [142] on cytological technique there are only a dozen or so paragraphs on this area. Information is now being

produced on these topics, in part by biochemists using glutaraldehyde as a probe of chemical structure.

Subramanian [143] showed that free ribosomes dissociated when they were centrifuged on a gradient. This could be prevented by glutaraldehyde. The tendency for interribosomal cross linking could be prevented by adding bovine serum albumin. Kahan and Kaltschmidt [144] have used glutaraldehyde to investigate the topographical relationships of the components of ribosomes of *Escherichia coli*. The ribosomes were exposed to glutaraldehyde and then their components separated by two-dimensional gel electrophoresis. Most of the reactive proteins were situated externally on the ribosomes.

Whole isolated chicken erythrocyte nuclei have been investigated by Olins and Wright [145]. Nuclei were allowed to react with different concentrations of glutaraldehyde and the histones separated electrophoretically; there was a decrease in the acid-soluble histones, apparently with pseudo-first-order rate constants, proportional to the lysine mode percentage of the fractions. This agrees well with the known reactions between protein–lysine and glutaraldehyde [24]. The polymerization of the histones was very rapid, the principal component being $F2C_n$ molecules where $n = 2$ to about 8. Olins and Wright [145] proposed that some F2C histones may exist in close proximity within the isolated nuclei.

Hopwood [146] has recently investigated the reaction of glutaraldehyde with nucleic acids. At temperatures up to 6°C no reaction occurs between native DNA and glutaraldehyde. At temperatures above 75°C the reaction follows pseudo-first-order kinetics, proceeding more rapidly at higher temperatures. The reactions between RNA and glutaraldehyde were similar, except that they began above 45°C. These findings were corroborated with thermal transition profiles. There was little evidence for the formation of intermolecular crosslinks, even at elevated temperatures.

These papers raise some interesting points. It would appear that under the normal conditions used for fixation, little reaction occurs between nucleic acids and glutaraldehyde, but rather that the fixative reacts with the histones and nuclear proteins. There is evidence that up to about 30% of cellular nucleic acids and nucleotides may be lost during fixation [147]. However, the tissue situation is more complex than the model one. Examination of $^{14}C$-formaldehyde-fixed soluble nucleohistone showed that two-thirds of the formaldehyde linkages were protein–protein bridges and a few of the remainder between proteins and nucleic acids. This

reaction of the nucleic acid may be related to the local structural alterations or breathing described by Printz and Von Hippel [149].

Woldringh [150] investigated the effect of sodium chloride on the organization of DNA during prefixation of *E.coli* cells with either 2.5% glutaraldehyde or 0.1% osmium tetroxide. Prefixation with glutaraldehyde resulted in a dispersed nucleoplasm, which was independent of the sodium chloride content of the growth medium. Presumably, the sodium chloride was unable to enter the cells, although a leakage of $K^+$ from the cells occurred during the glutaraldehyde prefixation. Woldringh thus concluded that a net loss of cations occurred, and that this cation effect might explain the dispersed state of the DNA.

## INTERACTION WITH LIPIDS

There is little information available on the reactions of glutaraldehyde and lipids, in spite of their structural importance in cells. Glutaraldehyde had little effect on the subsequent extraction of lipids from brains, compared with fresh unfixed controls. More recently, Nir and Hall [152] have compared the fixative effects of glutaraldehyde and formaldehyde in rod and cone membranes and also examined their fine structure. The aldehyde fixed material was exhaustively extracted with chloroform–methanol. Prefixation with glutaraldehyde rendered 38% of the phospholipids unextractable, whilst formaldehyde produced this change. These last workers showed, using thin-layer chromatography, that the phospholipids most dramatically effected were phosphatidyl ethanolamine and phosphatidyl serine. This is probably due to reaction with their amino groups, as Roozemond [153] postulated when he examined the phospholipids in rat hypothalamus.

Jost, Brooks and Griffith [154] compared the fluidity of phospholipid bilayers and neuron membranes after exposure to osmium tetroxide and glutaraldehyde with spin labelling. Osmium tetroxide abolishes the characteristic orientation on spin labelled bilayer regions and eliminates motion on electron spin resonance time scale. Glutaraldehyde reduces motion of maleimide spin labels covalently attached to protein, but has no effect on orientation and mobility in fluid bilayer regions and so does not prevent the potential for loss in phospholipid bilayer regions.

## INTERACTION WITH MICRO-ORGANISMS

Despite the fact that glutaraldehyde is known to be an important chemosterilizer, comparatively few studies have been made as to the way

in which it interacts with micro-organisms. It has already been pointed out (p. 282) that the aldehyde is more effective at alkaline than at acid pH, and that sodium bicarbonate is normally added to 'activate' the glutaraldehyde to the required pH (approx. 8) before use. Sodium bicarbonate itself possesses a bacteriostatic action against low numbers of *E.coli* but its inherent bactericidal activity is low – 0.3% w/v solution rendered 50% of the population of a washed suspension of *E.coli* (initial viable count $10^7$/ml) non-viable after 45 min at 37°C [155]. In relation to the antibacterial activity of alkaline glutaraldehyde, this is obviously of little consequence.

The primary effect of sodium bicarbonate may be on (i) the bacterial cell: bicarbonate treatment of washed suspensions of *E.coli* substantially reduced their absorbance (*A*) at 500 nm. This would appear to depend upon ionic strength rather than pH since the absorbance was lowered with increasing concentrations of bicarbonate whose pH remained between 7.5 and 8.5 [155]. This decrease in *A* was also evident with suspensions of *E.coli* envelopes, and with *E.coli* spheroplasts and *B. megaterium* protoplasts in an osmotically stabilized buffer [104, 156]; (ii) the glutaraldehyde molecule: at alkaline pH, glutaraldehyde gradually loses its activity. This inactivation was the result of polymerization of the molecule which occurred rapidly at pH 9 leading to an extensive loss of aldehyde groups [2]. However, bactericidal and sporicidal activity of glutaraldehyde (pH approx. 8–85) is measured in minutes and hours respectively, rather than in days or weeks, and accordingly the effect of sodium bicarbonate was considered by Munton and Russell [104] to be on the bacterial cell rather than on the aldehyde molecule. The electrophoretic mobility of *E.coli* cells is profoundly affected by increasing pH because of the ionization of surface groups to give an overall greater negative charge [157]. Support for this effect of bicarbonate [104] has been obtained from PMR studies [158].

Rubbo, Gardner and Webb [10] investigated the activity of various derivatives of glutaraldehyde against *B. subtilis* spores. Their findings show that an alteration in one or both aldehyde groups causes a complete loss of sporicidal activity, whereas substitution elsewhere in the molecule, i.e. with retention of the two free aldehyde groups, reduces the rate of kill but does not eliminate the activity. It is thus likely that the antibacterial activity is due to the two free aldehyde groups present. However, a comparison of the sporicidal activity of various aldehydes (*Table 4.2*, based on the data in [18, 20 and 108]) indicates a wide range in activity, and leads to the question as to why glutaraldehyde is the best of these substances. The answer to this must at present be rather specula-

Table 4.2. SPORICIDAL ACTIVITY OF SOME ALDEHYDES[ab]

| *Aldehyde*[c] | *Chemical structure* | *Sporicidal activity* |
|---|---|---|
| Formaldehyde (methanol) | HCHO | Good |
| Glyoxal (ethanedial) | CHO·CHO | Good[d] |
| Malonaldehyde (propanedial) | $CHO{\cdot}CH_2{\cdot}CHO$ | Slightly active |
| Succinaldehyde (butanedial) | $CHO{\cdot}(CH_2)_2{\cdot}CHO$ | Slightly active |
| Glutaraldehyde (pentanedial) | $CHO{\cdot}(CH_2)_3{\cdot}CHO$ | Excellent |
| Adipaldehyde (hexanedial) | $CHO{\cdot}(CH_2)_4{\cdot}CHO$ | Slightly active |

[a] Based on the data of Boucher, Last and Smith [18].

[b] Aldehydes beyond adipaldehyde have virtually no sporicidal activity.

[c] Names in parentheses are those recommended by the International Union of Pure and Applied Chemistry.

[d] Compare the results obtained by Pepper and Chandler [80], who suggest that glutaraldehyde is ten times as active as glyoxal, with succinaldehyde occupying an intermediate position.

tive, but if it is assumed that glutaraldehyde kills bacteria as a result of some form of protein interaction (see below also) then it could well be that the crosslinks formed with proteins are of the correct 'molecular length'. With adipaldehyde (hexanedial), $CHO{-}(CH_2)_4{-}CHO$, and aldehydes above this, the carbon chain length may be too great for effective crosslinking. Boucher, Last and Smith [18] postulate that the two predominant factors governing the biocidal activity of aldehydes are (i) the distance between the aldehyde groups and (ii) their tendency to polymerize. They argue that the fact that glutaraldehyde is the most potent resides in the differing tendency of these chemicals to polymerize. Thus, in glutaraldehyde the free aldehyde groups could be available to interact with the amino groups of the bacterial cell. Nevertheless, the actual site of reaction with the bacterial cell has yet to be fully elucidated, and it is obvious that such a reactive substance as glutaraldehyde could interact with several sites on or within the cell to bring about a lethal effect. Proven or possible sites are:

(a) Cell wall mucopeptide: Hughes and Thurman [159] have studied the effect of lysozyme, in the presence and absence of glutaraldehyde, on the isolated wall mucopeptide (murein, glycopeptide, peptidoglycan) from *B.subtilis* and found that glutaraldehyde-treated mucopeptide was less sensitive to lysis by lysozyme than untreated mucopeptide, although splitting of the lysozyme-sensitive bond by the enzyme still occurred.

They showed that approx. 30–50% of the available $-NH_2$ groups in isolated mucopeptide reacted with the dialdehyde, and they proposed that glutaraldehyde joined together two continuous side-chains in the case where both tripeptides carried free $\epsilon$-amino groups (*Figure 4.5*; bond A is the lysozyme-sensitive site). Presumably, a crosslink of this type would keep two adjacent tripeptides together even after cleavage at site A. This theory gains credence from the finding [159] that another crosslinking agent (1,5-difluoro-2,4-dinitrobenzene) substituted most of the free $-NH_2$ groups without, presumably, linking together the side-chains; thus, in this case the treated mucopeptide was solubilized by lysozyme as rapidly as the untreated control.

More recently, Russell and Vernon [160] have investigated the effect of lysostaphin on glutaraldehyde-treated whole cells, cell walls and isolated wall mucopeptide of *Staph. aureus*. Lysostaphin caused a rapid lysis of control whole cells, walls and (to a lesser extent, possibly as a result of

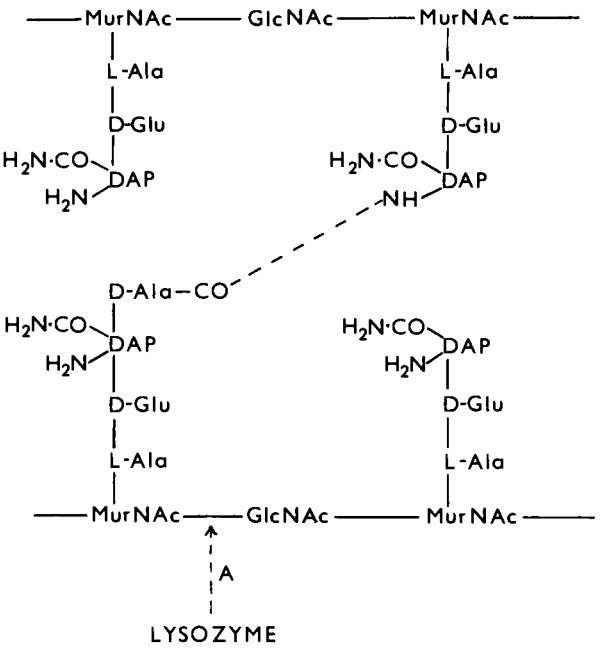



*Figure 4.5 Peptide side-chains in* B. subtilis *mucopeptide from Hughes and Thurman [159]). A is the linkage broken by lysozyme*

degradation during preparation) mucopeptide. Pretreatment of these with glutaraldehyde prevented this lysis.

Higgins, Pooley and Shockman [161] have found that glutaraldehyde has an effect on the cell wall of *Strep.faecalis* (strain ATCC 9790) which contained an autolysin existing in two forms: one latent and the other cell wall-bound and active. Glutaraldeyde was much more effective than osmium tetroxide or formaldehyde in blocking autolysis; although their results might suggest that the dialdehyde was effective because it was used at 30 times the concentration of osmium tetroxide, in fact glutaraldehyde at as low a concentration as 0.1% for only 5 min prevented autolysis.

(b) Cell wall protein: Recent studies with *E.coli* also indicate an effect of glutaraldehyde on the surface of Gram-negative bacteria [104, 162, 163]. Sodium lauryl sulphate (SLS) at 2% concentration at temperatures of 35–40°C and above causes a rapid dissolution of whole cells and walls of *E.coli*, possibly because of the extraction of lipoprotein from the wall. Pretreatment of cells and walls with glutaraldehyde, especially at pH 8 (as compared to pH 3) greatly reduced or completely inhibited subsequent SLS lysis, and this protection afforded by glutaraldehyde was greater with cells than with cell walls. McGucken and Woodside [164] have subsequently shown that glutaraldehyde reduces, but does not completely prevent, the liberation of reducing substances by hot acid from *E.coli* envelopes. As a result of studies with the enzymic activity of whole *E.coli* cells and disrupted cell preparations, Munton and Russell [165] suggested that the glutaraldehyde inhibition of cellular enzyme activity was a consequence of the interaction of the compound with the outer cell surface, which was thereby strengthened and in turn prevented ready access of substrate to enzyme. Strengthening by glutaraldehyde of the outer layers of spheroplasts thereby enabling them to withstand osmotic shock, has also been reported [163].

An unusual feature of the interaction of glutaraldehyde with microorganisms was the finding [166] that all the strains of Gram-negative bacteria tested became red in colour, whereas this did not happen with Gram-positive cocci, bacilli or spores, or with a mould or yeast (*Table* 4.3). A red colour was also produced with glutaraldehyde-treated penicillin-induced spheroplasts of *E.coli* which contain even less mucopeptide than do normal cells of this organism, and with isolated cell walls. The 'layer' responsible for this red colour development on treatment with glutaraldehyde is the lipoprotein moiety [166] which is present to a marked extent in the surface envelopes of Gram-negative, but not

Table 4.3. INTERACTION OF GLUTARALDEHYDE WITH SOME MICRO-ORGANISMS [166]

| *Organism* | *Culture or preparation* | *Colour development*[a] |
|---|---|---|
| *E. coli* | Whole cells | Red |
| | Penicillin-spheroplasts | Red |
| | Cell walls | Red |
| | Cytoplasmic constituents | Yellow |
| *K. aerogenes* | Whole cells | Red |
| *S. marcescens* | Whole cells | Red |
| *Pr. vulgaris* | Whole cells | Red |
| *Staph. aureus* | Whole cells | Slight yellow |
| *M. lysodeikticus* | Whole cells | None |
| *B. subtilis* | Whole cells (vegetative) | Yellow |
| *B. subtilis* | Fattened vegetative cells | Light yellow |
| *B. subtilis* | Spores | Light orange or yellow[b] |
| *B. megaterium* | Lysozyme-protoplasts | None |
| *Sacch. carlsbergensis* | Whole cells | None |
| *P. chrysogenum* | Whole cells | None |

[a] Exposure for 2 h at 37°C to 0.2% glutaraldehyde plus 0.3% sodium bicarbonate.
[b] After 24 h only.

Gram-positive, bacteria. The precise importance of this red colour is unknown, however, because (a) its visual appearance occurs long after death of Gram-negative bacteria takes place, and (b) Gram-positive bacteria, which do not become red in colour, are nevertheless highly sensitive to glutaraldehyde [166].

(c) Cytoplasmic membrane: Comparatively little attention has been devoted to this possible site of glutaraldehyde action. Ellar, Munoz and Salton [167] have shown that glutaraldehyde prevents the selective release of certain enzymes from the cytoplasmic membrane of *M.lysodeikticus* which normally occurs by controlled washing with buffers. In this context, formaldehyde will retard the release of $^{54}Mn$ after suspension of radioactive cells of *E.coli* in a non-radioactive medium [168], although whether this represents a 'sealing' of the wall or membrane, is unclear. Glutaraldehyde-treated lysozyme-induced protoplasts of *B.megaterium* become optically dense and resist lysis when transferred to a medium (water) of low osmotic pressure [156]. However, by their

very definition, protoplasts are wall-less forms and it is thus somewhat difficult to correlate this effect of glutaraldehyde with its lethal effect on whole cells.

(d) Bacterial spores: The mechanism by which bacterial spores are killed by glutaraldehyde is poorly understood. Boucher [108] considers that penetration of the aldehyde into the spore is necessary for sporicidal effect to be achieved, and support for this comes from Sykes [169] who suggests that spore permeability or impermeability to a chemical decides whether that chemical is, respectively, sporicidal or not.

Glutaraldehyde, however, is a highly reactive substance, and could interact strongly with the outer layers of the spores. The disulphide (S–S)-rich spore coat protein forms a structure which successfully masks hydrogen peroxide-reactive sites [170, 171]. Glutaraldehyde, at acid and alkaline pH, interacts to a considerable extent with the outer spore layers, presumably the spore coat(s), and this interaction reduces but does not completely prevent peroxide-induced (or lysozyme induced) lysis of thioglycollic acid, urea-sensitized spores. By interaction with the spore coat(s), glutaraldehyde could reduce the effectiveness of the thioglycollic acid plus urea although the dialdehyde interaction is likely to be with $-NH_2$ groups in the protein. However, an overall strengthening of the coat structure could result thereby preventing thioglycollic acid plus urea activity. Alternatively, of course, the thioglycollic acid plus urea could still cause rupture of H and S–S bonds, but glutaraldehyde cross-linking would prevent complete peroxide lysis. Disappointingly, our studies [83] with isolated spore coats have not given any satisfactory results, and further investigation is needed here.

Interestingly, although alkaline glutaraldehyde appears to have a somewhat greater effect than acid glutaraldehyde in reducing lysis of peroxide-treated thioglycollic acid plus urea-sensitized spores, the difference is slight in comparison to the relative sporicidal activities. This might suggest that acid glutaraldehyde interacts at the spore surface and remains there (thus causing marked changes in electrophoretic mobility [104]) whereas alkaline glutaraldehyde penetrates into the spore (thus causing smaller changes in electrophoretic mobility [104]) thereby producing a much greater sporicidal effect. It must be emphasised that this is, at present, only a tentative theory, that there is little experimental evidence in support, and that considerably more experimentation is needed for substantiation.

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# 5 The Chemistry and Biochemistry of C-Nucleosides

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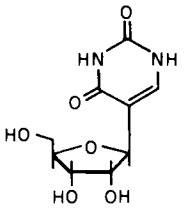
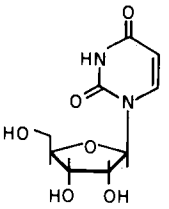
## INTRODUCTION

In 1959, during fractionation of RNA hydrolysates, Cohn isolated a fifth ribonucleoside in addition to the expected adenosine, guanosine, uridine and cytidine [1, 2]. This compound was identified first as 5-ribosyluracil and, later, definitively as 5-β-D-ribofuranosyluracil (pseudouridine, (1)) [3, 4]. Since this discovery, ten other naturally occurring C-nucleosides (six of which are antibiotics) have been isolated and characterized. Diverse biological effects, which include potentially useful antitumour and antiviral activities, have been exhibited by these C-nucleoside antibiotics.

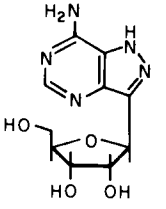
The C-nucleosides are striking in that the ribosidic linkage, a carbon–carbon bond rather than the usual nitrogen–carbon bond, is hydrolytically stable and therefore a potentially important pathway for catabolism of nucleosides or nucleotides [5, 6] – hydrolysis of the base from the sugar – does not affect these compounds. This enhanced stability undoubtedly has important consequences for the biochemical and medicinal potential of C-nucleosides as a class. Similarly important is the ability of C-nucleoside isosteres of metabolically functional N-nucleosides to serve as enzyme substrates. Thus pseudouridine (1) is recognized as uridine (2) at the nucleotide and polynucleotide levels [7, 8] and formycin (3) effectively replaces the isomeric adenosine (4) in reactions with a variety of enzymes



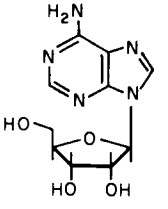
(1)



(2)



(3)



(4)

of intermediary metabolism, polynucleotide synthesis and tRNA synthesis and function [9–11].

The C-nucleoside literature has been selectively reviewed in this chapter. With respect to the naturally occurring C-nucleosides, most work published since 1970 is discussed; however only enough earlier material has been included to provide perspective since the excellent comprehensive works of Suhadolnik [9] and Hall [12] review results prior to this date. Earlier reviews deal with the chemistry [13] and biochemistry [14] of pseudouridine. Discussion of natural C-glycosyl compounds in which the carbohydrate is linked to other than a nitrogen heterocycle [15, 16] have been omitted. In reviewing work relevant to the synthesis of C-nucleosides, we have emphasized the various synthetic strategies which have been developed, though a more detailed discussion of this literature is available [17]. All biological data for synthetic C-nucleosides available to us have been included. Owing to the relatively short time synthetic efforts have been underway, these data are still somewhat meager. It is likely that much more biological data for synthetic C-nucleosides will be forthcoming during the next several years.

Our literature review was concluded in July 1975; however, some more recent material has been made available to us in prepublication form and has been included.

## PSEUDOURIDINE AND RELATED PYRIMIDINE C-NUCLEOSIDES

### PSEUDOURIDINE

Pseudouridine [5-($\beta$-D-ribofuranosyl)uracil*, $\psi$-uridine, $\psi$ (1), m.p. 223–224°C, p$K_a$ 8.9, $[\alpha]_D$-3.0° (c1% in $H_2O$)] appeared originally as an unknown nucleotide in an alkaline hydrolysate of calf liver RNA [3]. The structure of this 'fifth nucleotide' [4] was assigned by Cohn [2] as 5-ribosyluracil and the $\beta$-D-ribofuranosyl configuration was subsequently established [18]. Pseudouridine has been found in low molecular weight mRNA and in rRNA [19–25] and is particularly abundant in tRNA [26]. It

* In a strict sense designation of the C-nucleosides isomeric at C-1 of the carbohydrate moiety as $\alpha$- and $\beta$-anomers is incorrect; however, this designation is more readily understood than the systematic notation and is used throughout this review.

has been found in all tRNA species active in protein synthesis and occurs at specific positions of the nucleotide sequence [25–30]. Similarly, partial sequence analysis of rRNA obtained from a variety of sources revealed a non-random distribution of pseudouridine [19, 21, 31, 32]. Pseudouridine occurs with a frequency one-third that of uridine (2) in yeast and rat liver tRNA [25, 33].

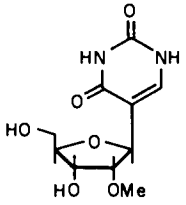
Pseudouridine appears to play a crucial role in tRNA mediated protein synthesis and may be involved in tRNA binding to the ribosome [34]. Glycine tRNA from *Staphylococcus epidermidis* lacks pseudouridine and is not active in protein synthesis [35]. Replacement of uridine-5'-triphosphate with the corresponding triphosphates of pseudouridine or 5-bromouridine (but not 5-fluorouridine) reduced the incorporation of leucine, tyrosine, lysine, isoleucine and valine into protein [36].

Normally, pseudouridine is excreted in the urine of man in an average amount of 66 mg/day. In case of malignant proliferation, the amount of excretion of pseudouridine increases [37], patients with acute leukemia, for example, excreting up to 167 mg/day. For those with acute granulocytic leukemia, the excretion may be up to 219 mg/day. However, patients with chronic lymphocytic leukemia excreted about 73 mg/day, which is considered within the normal range [38].

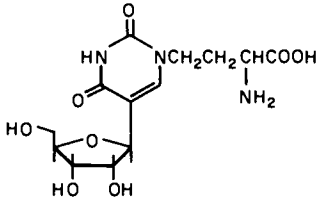
Radioactively labelled orotic acid has been injected into mice with resulting radioactivity found in pseudouridine in the urine. This incorporation of orotic acid into pseudouridine and pseudouridylic acid was strongly inhibited by the administration of 6-azauridine [39] (6-azauridine is known to block the conversion of orotidylic acid to uridylic acid in pyrimidine biosynthesis). It is of interest to note that 5-fluorouracil and methotrexate, which are inhibitors in other steps of pyrimidine biosynthesis, did not inhibit the formation of pseudouridine as did 6-azauridine. There is considerable evidence that uridine (2) is converted to pseudouridine (1) at the polynucleotide level [40, 41]. The discovery of enzymes which convert uracil and ribose-5-phosphate to pseudouridine-5'-monophosphate [42, 43] and kinases which convert pseudouridine (1) to the corresponding mono-, di-, and triphosphates [44] led to speculation that preformed pseudouridine monomer units are incorporated into RNA. However, more recently, Breitman [45, 46] has shown that pseudouridylate synthetase in pyrimidine auxotrophs of *E. coli* release uracil and ribose-5-phosphate for essential cellular reactions. These results suggest that the biochemical function of pseudouridylate synthetase is degradative.

## NATURALLY OCCURRING DERIVATIVES OF PSEUDOURIDINE

C-Nucleosides structurally related to pseudouridine have been isolated and identified Hall [47] isolated 2′-*O*-methylpseudouridine, 5-(2′-*O*-methyl-β-D-ribofuranosyl)uracil (5), from an enzymatic digest of yeast tRNA. The initial amount obtained did not permit a rigorous structure identification and evidence for its existence rested solely on spectral and chromatographic behavior of the isolated sample. Hudson, Gray and Lane [48] obtained this compound as the nucleotide from wheat germ tRNA and more recently, Gray [49] found it to be a constituent of wheat embryo cystosol rRNA. The 5′-monophosphate was isolated from snake venom hydrolysates of the 18 S + 26 S ribonucleates of wheat embryo. Also, 2′-*O*-methylpseudouridine (5) has been identified in a specific sequence of serine tRNA from rat liver [50].




(5)



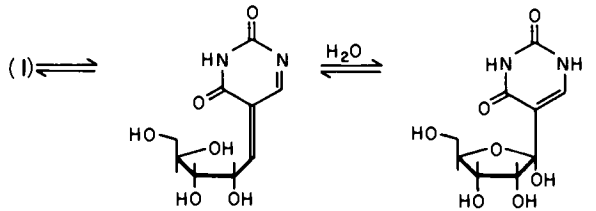

(6)

Saponara and Enger [51] isolated the hypermodified pseudouridine derivative, 1-methyl-3-(3′-amino-3′-carboxypropyl)-pseudouridine (6), from a hydrolysate of the 18 S rRNA of Chinese hamster cells. One of four uracil-bound amino acids detected in germinating pea seedlings has been assigned the structure 1-alanyl-5-ribosyl-4-aminouracil [52]; however, the data presented are fragmentary and not convincing.

## STRUCTURAL MODIFICATIONS OF PSEUDOURIDINE

In the process of elucidating the structure of pseudouridine, a number of derivatives were prepared [13]. Thus, it was discovered that, in the presence of acid or base, pseudouridine undergoes isomerization at C-1′ yielding a mixture of the 5-ribofuranosyl- and 5-ribopyranosyluracil anomer pairs [13, 53, 54]. This isomerization of the carbohydrate moiety, which has not been observed for N-nucleosides, has been attributed [55] to the intermediacy of the open-chain isomer (7), accessible owing to the

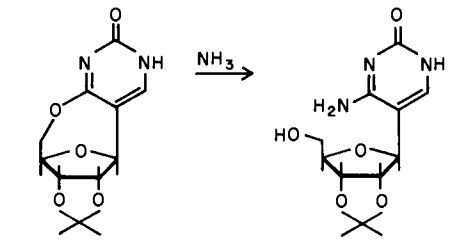
allylic character of C-1′ of pseudouridine. Similarly, the initially surprising result that periodate oxidation of (1) yields 5-formyluracil has been explained by invoking the intermediacy of (8) formed by addition of water to (7) followed by cyclization [13, 55]. Exposure of pseudouridylic acid to 254 nm light also yielded 5-formyluracil [56]. Attempts to utilize the unique sensitivity of pseudouridine to oxidation in order to selectively cleave rRNA have achieved only limited success [13, 55, 56].



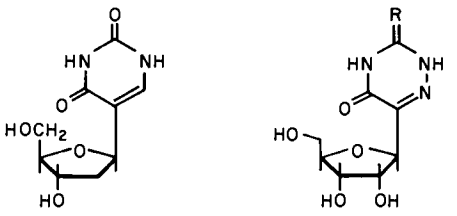

(7) (8)

Pseudouridine undergoes facile alkylation at both N-1 and N-3. Thus, treatment of pseudouridine with excess *N*-cyclohexyl-*N*′-β-(4-methylmorpholinium)ethylcarbodi-imide *p*-toluenesulfonate, in aqueous solution at pH 9, yielded either a mixture of N-1 and N-3 alkylated products or, with longer reaction times, a mixture of the 1,3-disubstituted compound and the stable N-3 substituted compound. This latter compound is formed by dealkylation of the 1,3-disubstituted derivative which is unstable at the pH of the reaction [57]. Similarly, acrylonitrile reacts with pseudouridine to give either the 1-cyanoethyl- or the 1,3-bis-cyanoethyl derivative [53, 58, 59]. Reactions of acrylonitrile [60] or the water-soluble carbodi-imide [57] under controlled conditions are selective for pseudouridine and can be used for specific alkylation of RNA. In earlier work, 1-methyl-, 3-methyl- and 1,3-dimethylpseudouridine were prepared [2, 61].

Michelson and Cohn [18] prepared *O*-4:*C*-5′-cyclo-2′,3′-*O*-isopropylidene pseudouridine (9) by treatment of the intermediate 2′,3′-*O*-isopropylidene-5′-*p*-toluenesulfonate with sodium t-butoxide. Treatment of this compound (9) with ammonia yielded 2′,3′-*O*-isopropylidene-5-β-D-ribofuranosylcytosine (10) [18]. Using a partially purified pseudouridylate synthetase, Kalman [62] converted uracil and 2-deoxyribose-5-phosphate to 2′-deoxypseudouridylic acid which, upon incubation with snake venom 5′-nucleotidase (EC 3.1.3.5), yielded 2′-deoxypseudouridine (11). Cyanoethylation of (11) yielded the corresponding N-1 cyanoethyl derivative. 2′-Deoxypseudouridylic acid is an effec-




(9) (10)




(11) (12)

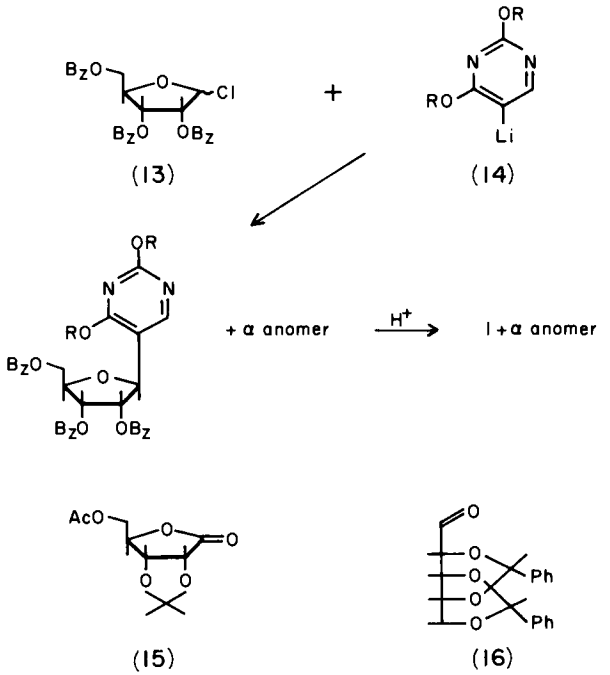
tive inhibitor of thymidylate synthetase, competitive with the substrate, 2'-deoxyuridylic acid [62].

Treatment of 2',3',5'-tri-*O*-acetylpseudouridine with ozone resulted in degradation of the pyrimidine ring to a two carbon fragment (C-4 and C-5) which, upon condensation with thiosemicarbazide and hydrolysis of the acetyl esters, yielded 6-(β-ribofuranosyl)-3-thio-1,2,4-triazin-5-one (12,R = S) [63, 64]. This compound was then methylated on sulphur and hydrolyzed in acid to yield 6-azapseudouridine (12, R = O).

## SYNTHESIS OF PSEUDOURIDINE

Pseudouridine and its α anomer were synthesized by Shapiro and Chambers [65], albeit in very low yield (2% and 1% respectively), by condensation of 2,3,5-tri-*O*-benzoyl-D-ribofuranosyl chloride (13) with 2,4-dimethoxypyrimidin-5-yl lithium (14, R = Me) followed by separation of anomers and hydrolysis of the protecting groups.

Asbun and Binkley [66] prepared pseudouridine in 10% overall yield by the condensation of 2,4-bis(benzyloxy)pyrimidin-5-yl lithium (14, R = benzyl) and 5-*O*-acetyl-2,3-*O*-isopropylidine-D-ribonolactone (15) followed by sodium borohydride reduction, catalytic hydrogenation and treatment with acid. Condensation of 14 (R = benzyl, t-butyl) with

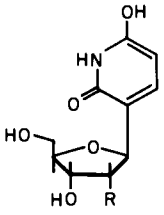



2,4:3,5-di-*O*-benzylidene-*aldehydo*-D-ribose (16) [67], or the corresponding di-*O*-isopropylidene derivative [68] followed by treatment of the resulting epimeric alcohol mixture with methanolic hydrogen chloride produced pseudouridine in improved (18%) yield. The yield of pseudouridine prepared by this general route was further improved by use of milder, more selective reaction conditions [69].

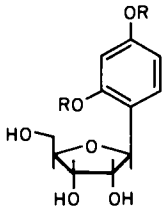
## SYNTHETIC C-NUCLEOSIDES RELATED TO PSEUDOURIDINE

The preparation of a number of C-nucleoside analogues of pseudouridine have been reported (see *Table 5.1*) [63–78]. Using synthetic sequences similar to that used for the preparation of pseudouridine, 5-β-D-ribofuranosylcytosine [70] obtained earlier [18] as the isopropylidene derivative (10), and ring analogues (17–19) (71, 72) were prepared. 1-Deazapseudouridine (3-D-ribofuranosyl-2,6-dihydroxypyridine (17) and the 2′-deoxy analogue (18) were tested [71] for inhibition of thymidylate synthetase with no detected activity. This result is not surprising since the 5′-phosphate is an essential feature of inhibitors of this enzyme. These

compounds also failed to inhibit growth of *E. coli* B/r. The dideaza analogue of pseudouridine (19), more recently prepared [72], failed to inhibit the growth of *S. faecium* or *E. coli*. However, a synthetic precursor, the 2,4-dibenzyl derivative of dideazapseudouridine (20), inhibited the growth of leukemia L1210 cells by 50% at $7 \times 10^{-6}$ M and that of mammary carcinoma $TA_3$ cells at $5 \times 10^{-5}$ M. Dideazapseudouridine (19) itself inhibited L1210 at $10^{-4}$ M but was not effective against $TA_3$ cells [72]. The inhibition of L1210 by the dibenzyl derivative (20) was reversed competitively by uracil, uridine and 2'-deoxyuridine. 1,3-Dideazapseudouridine (19) at $3 \times 10^{-5}$ M decreased the plaque diameter of herpes simplex virus (type 1) by 50%; at $10^{-4}$ M, plaque formation was reduced by over 90%.



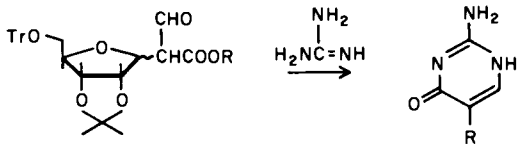
(17) R = OH
(18) R = H



(19) R = H
(20) R = benzyl

Chu, Watanabe and Fox [73] condensed methyl or ethyl 2-(2',3'-isopropylidene-5'-trityl-D-ribofuranosyl)-2-formylacetate (21) with guanidine to form a single nucleosidic product in 30% yield which, by treatment with acid, gave a readily separable mixture of the isomeric free nucleosides (22a and 22b). Using closely related synthetic routes, a number of other 5- [74] and 6-ribofuranosylpyrimidines [75] have been prepared (see *Table 5.1*).

In preliminary studies 2-amino-5-(β-D-ribofuranosyl)-4-pyrimidinone (pseudocytidine, 22a) at a concentration of 0.8 μg/ml inhibited the growth



(21) (22)

a, β-D-ribofuranosyl
b, α-D-ribofuranosyl

Table 5.1. SYNTHETIC C-NUCLEOSIDES RELATED TO PSEUDOURIDINE

| *Compound* | *References* |
|---|---|
| Uracil, 5-α-D-ribofuranosyl- | [65–69] |
| 1,5-bis-α-D-ribofuranosyl- | [67] |
| 5-α-D-mannitol- | [66] |
| 5-β-D-gulitol- | [66] |
| 5-β-D-gulopyranosyl- | [66] |
| 5-α-D-arabinitol- | [68] |
| 5-(D-allopentahydroxypentyl)- | [68, 69] |
| 5-(D-altropentahydroxypentyl)- | [69] |
| 5-β-D-xylofuranosyl- | [68] |
| 5(2′,3′,5′-tri-O-acetyl-β-D-arabinofuranosyl- | [68] |
| 5-(D-*altro*-1-acetamido-2,3-4,5-tetrahydroxypentyl)- | [69] |
| Cytosine, 5-β-D-ribofuranosyl- | [70] |
| 5-α-D-ribofuranosyl | [70] |
| 2-amino-4-hydroxypyrimidine(isocytosine), | |
| 5-β-D-ribofuranosyl- | [73] |
| 5-α-D-ribofuranosyl- | [73] |
| 6-β-D-ribofuranosyl-[a] | [75] |
| 2-Hydroxy-4-thiopyrimidine, | |
| 6-β-D-ribofuranosyl-[a] | [75] |
| 6-β-D-ribofuranosyl-5-carbethoxy-[a] | [75] |
| 6-α-D-ribofuranosyl-5-carbethoxy-[a] | [75] |
| 2,4,6-Trihydroxypyrimidine, 5-β-D-ribofuranosyl-[a] | [74] |
| 2,6-Dihydroxypyridine (3-deazauracil), 3-D-ribofuranosyl- | [71] |
| 3-(2′-deoxy-D-ribofuranosyl)- | [71] |
| 1,3-Dihydroxybenzene (1,3-dideazauracil), | |
| 4-β-D-ribofuranosyl- | [72] |
| 3,5-Dihydroxy-1,2,4-triazine (6-azauracil)[b], | |
| 6-β-D-ribofuranosyl- | [63, 64] |
| 6-(1,4-anhydro-D-ribo-tetrahydroxybutyl)- | [76] |
| 6-(1,4-anhydro-L-lyxo-tetrahydroxybutyl)- | [76] |
| 6-(1,4-anhydro-D-lyxo-tetrahydroxybutyl)- | [76] |
| 6-(L-xylo-tetrahydroxybutyl)- | [76] |
| 6-(D-arabino-tetrahydroxybutyl)- | [76] |
| 6-(D-ribo-tetrahydroxybutyl)- | [76] |
| 6-(D-allo-pentahydroxypentyl)- | [77] |
| 6-(D-altro-pentahydroxypentyl)- | [77] |
| 6-(D-gluco-pentahydroxypentyl)- | [77] |
| 6-(D-galacto-pentahydroxypentyl)- | [77] |
| 6-(2,5-anhydro-D-allo-pentahydroxypentyl)-[c] | [77] |
| 6-(2,5-anhydro-D-altro-pentahydroxypentyl)- | [77] |

[a] Only the 2′,3′-isopropylidene-5′-trityl derivative has been reported.

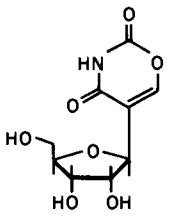
[b] In addition to those compounds listed in this table references 76 and 77 report the .

of leukemia 5178 Y cells in vitro to the extent of 50%. Under similar conditions the $ID_{50}$ value for the closely related nucleoside antibiotic, 5-azacytidine, is 1.0 $\mu$g/ml. This inhibition by pseudocytidine (22a), like that by 5-azacytidine, is blocked by uridine and cytidine but is not affected by 2'-deoxycytidine. The $\alpha$-anomer (22b) is a considerably less effective growth inhibitor of leukemia 5178 Y cells [73].

Bobek, Farkas and Sorm [63, 64, 76–78] have synthesized more than twenty-five 1,2,4-triazine C-nucleosides (see *Table 5.1*). No biological test data for these interesting compounds are available.

## OXAZINOMYCIN (MINIMYCIN)

Oxazinomycin [5-$\beta$-D-ribofuranosyl-1,3-oxazin-2,4-dione, minimycin (23), m.p. 164–166°C (dec.), p$K_a$ 6.96, $[\alpha]_D^{20}$ + 19.7° (c 1% in $H_2O$)] was isolated in Japan from *Streptomyces tanesashinensis* nov. sp. [79]. A second Japanese group [80, 81] isolated this same antibiotic, which they called minimycin, from a Streptomyces strain sp. 80432. Oxazinomycin (23) is effective against both Gram-positive and Gram-negative bacteria, particularly against *Staphylococcus aureus* and exhibits some activity against yeast (*Candida albicans*) and fungi (*Trichophyton interdigitale*) [79]. Antitumour activity against Ehrlich ascites carcinoma in mice [79] and solid tumour cells in vitro [82] has been reported. In mice, the $LD_{50}$ value was 10–20 mg/kg (intraperitoneally) and 100–120 mg/kg (intravenously) [79].




(23)

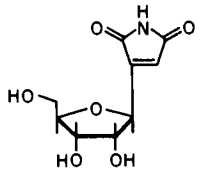
preparation of a number of thio-, methylthio- and N-methyl-analogues of these 1,2,4-triazine C-nucleosides.

[c] Initially this compound was erroneously assigned [78] the structure 6-($\beta$-D-ribofuranosyl)-3,5-dihydroxy-1,2,4-triazine (6-azapseudouridine)(12, R = O)[63, 64].

Oxazinomycin is converted to pseudouridine upon treatment with methanolic ammonia and is biogenetically related to showdomycin [82].

## SHOWDOMYCIN

Showdomycin [3-(β-D-ribofuranosyl)-2,5-pyrroledione, 2-(β-D-ribofuranosyl)maleimide (24), m.p. 160–161°C, $pK_a$ 9.29, $[\alpha]_D^{22.5} + 49.9°$ (c 1% in $H_2O$), $\lambda_{max}$ 220 nm], was isolated in 1964 from *Streptomyces showdoensis* [83]. The yield of the nucleoside antibiotic was about 400 μg/ml. Subsequently, it was found that a mutant of *S. showdoensis*, isolated from the parent strain following treatment with 4-nitroquinoline 1-oxide, was a much better source of showdomycin (1 mg/ml) in synthetic medium [84, 85].




(24)

The structure of showdomycin (24), which resembles both that of pseudouridine and uridine, was elucidated independently by the research group at the Shionogi Research Laboratories [86] in Japan and by Darnall, Townsend and Robins [87, 88] in the United States. Elucidation of the structure of showdomycin, accomplished through spectrometric and chemical studies (reviewed by Suhadolnik [9]) has been confirmed by X-ray analysis [89].

Biosynthesis of showdomycin in *S. showdoensis* involves conversion of fumarate or succinate by the Krebs cycle and the malic enzyme. Proof that C-4 of glutamate or α-ketoglutarate forms the carbon–carbon bond with D-ribose was obtained when [5-$^{14}$C,4-$^{3}$H]glutamate was incorporated into showdomycin with retention of $^{14}$C in the maleimide ring and loss of tritium. Therefore, the four-carbon maleimide ring arising from C-2 to C-5 of α-ketoglutarate must be an asymmetric unit when condensation occurs with D-ribose [84, 85, 90].

A number of biochemical properties of showdomycin has been described. In *E. coli*, showdomycin causes a massive biosynthesis of RNAs which are not complementary to DNA [91]. Komatsu and Tanaka

[92] studied the action of showdomycin on the incorporation of amino acids and purine and pyrimidine bases into protein and nucleic acids in *E. coli* K-12 cells. More recently, they have shown [93] that showdomycin (24) strongly inhibits the uptake of deoxycytidine by membrane vesicles of *E. coli* K12 cells. Also, showdomycin produced a rapid efflux of deoxycytidine from previously loaded *E. coli* K12 vesicles.

Kalman [94] demonstrated that showdomycin irreversibly inactivated thymidylate synthetase of *L. casei*. Prior treatment of the enzyme with hydroxyethyldisulfide or addition of the enzyme substrate deoxyuridylate prevented the inhibitory effect. Reduction of the 3,4 double-bond of showdomycin (24) resulted in loss of inhibitory activity. 5′-Phosphorylation greatly enhanced the inhibitory effect. These results suggest that the maleimide ring of showdomycin alkylates a sulphydryl group at the active site of thymidylate synthetase. In similar experiments, Tobin and Akera [95] have shown that showdomycin is a nucleotide-site-directed inhibitor of $(Na^+ + K^+)$ATPase and have concluded that this inhibition is due to reaction of showdomycin with a sulphydryl group at the nucleotide-binding site of the enzyme. Showdomycin is also an inhibitor of UMP kinase and uridine phosphorylase but does not inhibit uridine kinase [96].

The radiosensitization of *E. coli* B/r cells by showdomycin, as reported by Titani and Katsube [97, 98], indicates that showdomycin can be useful in studying the mechanism of the sensitization phenomenon since showdomycin is a novel thiol-binding agent and this antibiotic is less toxic to *E. coli* B/r than are other known thiol-binding compounds. Showdomycin was found to enhance the lethal effect of alkylation ('radiomimetics sensitization') of *E. coli* B/r when combined with biological alkylating agents. Although showdomycin alone did not show any serious toxicity to cells, a combination of showdomycin with either 2,2′-dichlorodiethylamine (nor-$NH_2$) or methyl methanesulfonate (MMS) considerably enhanced the lethal effects of these two alkylating agents in spite of their different modes of alkylation [97, 98].

A study using showdomycin analogues indicated that the enhanced susceptibility of *E. coli* B/r to alkylating agents is specific not only for the thiol-binding ability but also for structural properties of the thiol-binding agent [99]. Using showdomycin, showdomycin 2′,3′,5′-triacetate, 2′,3′-isopropylidine showdomycin and *N*-hydroxymethylshowdomycin in studies under anoxic and aerated conditions, it was found that the radiosensitizing ability of these compounds under anoxic conditions is mainly derived from the maleimide moiety, and the mode of action is

similar to that of *N*-ethylmaleimide. In contrast, when the system was aerated, the hydroxyl groups of the sugar moiety, which are essential to the antibiotic activity of showdomycin, also seem to be essential for its radiosensitizing ability [100].

Although certain chemical properties of showdomycin and *N*-ethylmaleimide are similar, their biological properties are rather different. Another maleimide derivative, 3-methylmaleimide (citraconimide), differs both in chemical and biological activity from either showdomycin or *N*-ethylmaleimide [101].

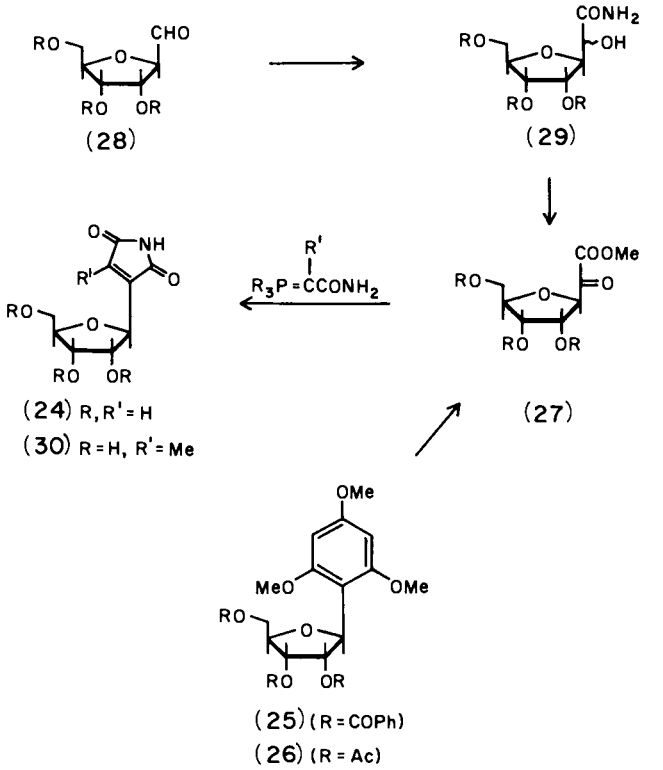
Showdomycin is a broad spectrum antibiotic which is active against both Gram-positive and Gram-negative bacteria, especially against *Streptococcus hemolyticus* and *S. pyogenes.* It shows remarkable activity against Ehrlich ascites tumor in mice and cultured HeLa cells [83, 102]. Showdomycin is less active against ascites hepatoma AH-130 in Wistar rats and is inactive against leukemia L1210, Walter 256 carcinosarcoma, sarcoma 180, and Lewis lung cancer. Some toxicity has also been noticed [103]. It is more toxic in mice when administered intraperitoneally or subcutaneously than when administrated intravenously [83].

## SYNTHESIS OF SHOWDOMYCIN

Two syntheses of showdomycin have been reported. Kalvoda, Farkas and Sorm [104] condensed 2,3,5-tri-*O*-benzoyl-D-ribofuranosyl bromide with 1,3,5-trimethoxybenzene and the resulting C-nucleoside (25) was hydrolyzed and re-esterified to 1-(2,3,5-tri-*O*-acetyl-$\beta$-D-ribofuranosyl)-2,4,6-trimethoxybenzene (26) which, upon ozonolysis, yielded the key intermediate, ketoester (27, R = Ac). Trummlitz and Moffatt [105] converted 2,5-anhydro-3,4,6-tri-*O*-benzyl-D-allose (28, R = $CH_2Ph$) to 3,6-anhydro-4,5,7-tri-*O*-benzyl-D-glycero-D-*allo*heptonamide and its D-glycero-D-altro isomer (29). Methanolysis of these substances and oxidation afforded (27, R = benzyl); i.e., essentially the same intermediate prepared by the Czech group [104].

For construction of the maleimide ring, both research groups [104, 105] reacted ketoester (27) with a Wittig reagent. When carbamoylmethylenetriphenylphosphorane was used [105], the desired maleimide ring was formed directly and hydrolysis of the carbohydrate blocking groups yielded showdomycin (24). Use of ethoxycarbonylmethylenetriphenylphosphorane [104] proved a more lengthy route requiring intermediate formation of the maleic anhydride, ring opening with ammonia and subsequent recyclization.

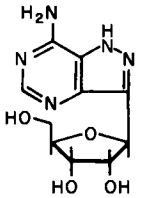
Trummlitz, Repke and Moffatt [106] have extended their route by modifying the phosphorane used for reaction with ketoester (27) to synthesize 3-methylshowdomycin (30). The mass and NMR spectra of (30) are quite similar to those of showdomycin (24); however, the optical rotatory dispersion of the two compounds are different. 3-Methylshowdomycin (30) exhibits a negative rotation and a negative optical rotatory dispersion spectrum centered about 386 nm while showdomycin is dextrorotatory [107] and shows a positive Cotton effect. The authors [106] suggest that these differences are indicative that the ring methyl group of (30) leads to an inversion of the normal *anti* glycosyl conformation and that (30) possesses a stable *syn* conformation. The possibility that the optical properties of (30) indicated it to be an $\alpha$-nucleoside, while not conclusively ruled out, was deemed unlikely based on the method of synthesis and the well-studied properties of precursor (27).



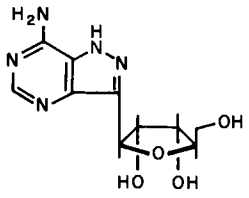
## THE FORMYCINS

### FORMYCIN

Formycin [7-amino-3-(β-D-ribofuranosyl)pyrazolo[4,3-*d*]pyrimidine, formycin A (3), m.p. 141–144°C (dec. > 252°C), p$K_a$ 4.5 and 9.5, $[\alpha]_D^{25}$–35.5° (c 1% in 0.1 N HCl), $\lambda_{max}^{pH\,1}$ 234, 295 nm, $\lambda_{max}^{pH\,11}$ 235, 305 nm] was initially isolated from the culture filtrate of *Norcardia interforma* [108] and later from culture broth of *Streptomyces lavendulae* [109] and *S. gummaenis* [110]. The structure of formycin (3) was established through degradation studies, by UV and NMR spectral interpretations, and by X-ray crystallography [111, 112]. In addition to the gross structural information, the X-ray crystallographic study [112] revealed two features of the formycin structure which have achieved great significance as its biological effects have been investigated. First, the C–C glycosidic bond in formycin is 1.55 Å in length, significantly longer than the 1.47 Å bond length of a C-N glycosidic linkage, and secondly, formycin hydrobromide exists in *syn* conformation (3a) rather than *anti* (3b) as is the case for adenosine (4). More recently, a crystallographic study [113] of formycin hydrate revealed that this neutral species exhibited a glycosyl torsion angle ($\phi$) of 109.8° which is intermediate between classical *syn* and *anti* torsion angles. The striking differences in conformational preferences shown by formycin and adenosine have been the subject of a molecular orbital study [114].




(3a) syn




(3b) anti

In an elegant series of investigations, Ward and Reich [115–118] elaborated important implications of the increased conformational flexibility of formycin for its biological effects. Since these studies have been reviewed elsewhere [9–11], it is sufficient to note that formycin can substitute for adenosine in numerous enzymatic reactions [118]. Among these are adenosine deaminase (EC 3.5.4.4) which is known to require nucleoside substrates of *anti* conformation [119]. Conversely, polyformycin is facilely degraded by pancreatic ribonuclease, a 'pyrimidine-specific'

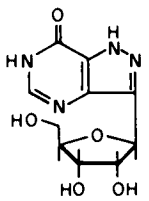
enzyme, but is highly resistant to degradation by nonspecific nucleases which readily degrade polyadenosine. This anomaly has been rationalized [11, 115–118] as a result of the *syn* conformation of formycin residues in polyformycin which have the potential for replacing a pyrimidine in nucleic acid polymer hydrogen bonding. More recently, polyformycin and polyformycin B have been shown to form complexes with both an oligomer of cycloadenylic acid ($\phi_{CN} = 120°$) and with polymers of natural nucleotides ($\phi_{CN} = 0°$) further emphasizing the flexibility in torsion angles of the formycins [120].

Formycin exhibits activity against Ehrlich carcinoma in mice, Yoshida rat sarcoma cells, HeLa cells, influenza virus A-1 in cells of chick choiallantoic membrane, *Xanthomonas oryzae* and *Mycobacterium* 607 [108, 121–126]. This antibiotic was shown to inhibit purine metabolism and, more specifically, phosphoribosylpyrophosphate biosynthesis [127, 128].

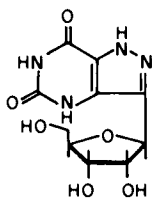
## FORMYCIN B AND OXOFORMYCIN

Formycin B [3-($\beta$-D-ribofuranosyl)pyrazolo[4,3-*d*]pyrimidine, laurusin (31), m.p. 254–255°C, $[\alpha]_D^{20}$–51.5° (c 1% in $H_2O$), $\lambda_{max}^{pH1}$ 221, 276 nm, $\lambda_{max}^{pH7}$ 219, 280 nm, $\lambda_{max}^{pH11}$ 230, 292 nm] was isolated from culture filtrates of *Nocardia interforma* [129] and *Streptomyces lavendulae* [109]. The structure elucidation of formycin B (31) [111, 112, 130] followed closely that of formycin as it was recognized [111] that hydrolysis of the amino function of formycin yielded formycin B.

Successful deamination of formycin to formycin B [111] using adenosine deaminase provided additional evidence that these two antibiotics are $\beta$-nucleosides as LePage and Junga [131] reported that this enzyme utilizes only the $\beta$-isomers of adenosine analogues and does not attack $\alpha$-isomers. The reverse enzymatic reaction, amination of formycin B to produce formycin, has also been reported [132] and suggests that the biosynthesis of formycin involves amination of formycin B 5′-monophosphate. Working with *Streptomyces sp.* MA406-A-1, the effects



(31)



(32)

of addition of various amino acids on the production of formycin were examined [133]. Lysine, asparate or glutamate stimulates the production of formycin. The suggestion was made [133] that biosynthesis of formycin in this organism is closely related to lysine metabolism and is not related to biosynthesis of purine nucleosides.

Formycin B is readily oxidized to oxoformycin (oxoformycin B, 32), a xanthosine analogue, by liver aldehyde oxidase [134] and is excreted in this form into the urine of mice and rabbits [132, 135, 136]. Conversion of formycin B to oxoformycin also occurs in *N. interforma*, *P. fluorescens* and *S. kasugaensis* [132]. Both formycin and formycin B are competitive inhibitors of xanthine oxidase. These effects exemplify an important biological difference between the formycins and their purine counterparts since neither adenosine nor inosine is a substrate or inhibitor of xanthine oxidase (EC 1.2.3.2).

Formycin B is a potent competitive inhibitor of purified human erythrocytic nucleoside phosphorylase. It also inhibits the degradation of nucleosides by intact and hemolyzed human erythrocytes and by sarcoma 180 ascites cells [135]. Changes in morphogenetic pathways in *Dictyostelium discoidem* under the influence of formycin B were also studied [137]. Formycin B inhibited *X. oryzae* and *P. filamentosa* [109, 129]. Formycin B did not inhibit fungi, HeLa cells, Yoshida rat sarcoma, Ehrlich carcinoma or leukemia L1210 [122, 136]. It has shown activity against influenza virus [125, 138] and was inhibitory toward tobacco mosaic virus multiplication without causing phytotoxicity to host plants [139].

## STRUCTURAL MODIFICATIONS OF FORMYCIN

### *7-Substituted derivatives*

A number of 7-substituted-3-$\beta$-D-ribofuranosylpyrazolo[4,3-*d*]pyrimidines have been prepared from formycin B [130, 140, 148] and limited biological test data for these compounds has been reported (see *Table 5.2*). While various of the compounds proved inhibitory in certain test systems, these data include no impressive biological activities. Particularly disappointing has been the failure of 7-thio- and 7-alkylthio-compounds, i.e., analogues of the very active 6-thiopurine nucleosides [5], to exhibit appreciable antitumour activity [125, 130, 138]. However, since the available data are quite meager, considerably more test data will be required to adequately explore the medicinal (particularly antitumour and

Table 5.2. 7-SUBSTITUTED-3-β-D-RIBOFURANOSYLPYRAZOLO[4,3-*d*]-PYRIMIDINES. DERIVED FROM THE FORMYCINS

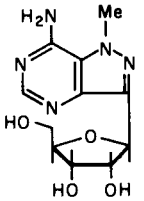
| *7-Substituent* | *Biological activity* | *Reference* |
|---|---|---|
| NHMe | Yoshida sarcoma (–)[a], *X. oryzae* (+), influenza virus (–), *S. aureus* (+), *C. albicans* (+), *E. aroideae* (+), *Mycobacterium* 607 (+), *M. tuberculosis* BCG (+) | [130, 138] |
| $NMe_2$ | Yoshida sarcoma (–), *X. oryzae* (+), influenza virus (–), *S. aureus* (+), *C. albicans* (+), *E. aroideae* (+), *Mycobacterium* 607 (+), *M. tuberculosis* (+) | [130, 138] |
| $NMe_3^+$ | | [140] |
| NHOH | Yoshida sarcoma (+), *X. oryzae* (+), *S. aureus* (+), *S. cerevisae* (+), *Mycobacterium* 607 (+), *M. tuberculosis* (+) | [130, 138, 140] |
| $NHNH_2$ | *S. aureus* (+) | [130, 140] |
| morpholino | *S. aureus* (+), *E. aroideae* (+) | [130] |
| $NHCH_2Ph$ | | [141] |
| NH-isopentenyl | Human leukemia cell line (+) | [141] |
| $N{=}CHNMe_2$ | | [142] |
| H | Yoshida sarcoma (+), *X. oryzae* (–), influenza virus (–) | [125, 130, 143] |
| Cl | Yoshida sarcoma (+), *X. oryzae* (+), influenza virus (+), Ehrlich ascites (–) | [125, 130] [138, 140] |
| Br | *X. oryzae* (+), Ehrlich ascites (–) | [130] |
| I | Influenza virus (+), *X. oryzae* (+), Ehrlich ascites (–) | [125, 130, 138] |
| SH | Yoshida sarcoma (+), *X. oryzae* (–), influenza virus (+), *S. aureus* (+), *E. aroideae* (+), Ehrlich ascites (–) | [125, 130, 138, 140] |
| SMe | Yoshida sarcoma (+), *X. oryzae* (–), influenza virus (+), *S. aureus* (+), *E. aroideae* (+) | [125, 130, 138, 140] |
| $SO_3H$ | | [130] |
| SCOOEt | | [130] |
| $SCH_2CH{=}CH_2$ | | [140] |
| SeH | | [143, 144] |
| SeMe | | [143] |
| $SeCH_2Ph$ | | [143] |
| $SeCH_2(p\text{-}O_2NPh)$ | | [143] |
| $OCH_3$ | | [143] |

[a]Inhibition was observed when conformycin [145–147], an adenosine deaminase inhibitor, was added.

antiviral) potential of base substituted analogues of formycin. It appears particularly desirable to extend synthetic efforts to include 5,7-disubstituted compounds (formally, derivatives of oxoformycin) so that these derivatives, also, can be tested for biological activities.

*Nitrogen-substituted derivatives*

Treatment of formycin with methyl iodine under basic conditions produced a mixture of 1-methyl- (33) and 2-methyl- (34) formycins in a ratio of approximately 1:6 [149]. Methylation of formycin with dimethylformamide dimethylacetal and ammonium hydroxide yielded the 1-methyl isomer (33) selectively [142]. Somewhat surprisingly, definitive assignment of the sites of alkylation of (33) and (34) proved difficult and was accomplished using magnetic circular dichroism [149] and X-ray crystallography [150]. When (34) was treated with 1 N sodium hydroxide under reflux, 2-methylformycin B was produced [149].

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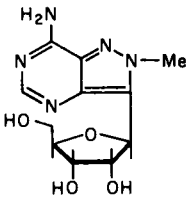
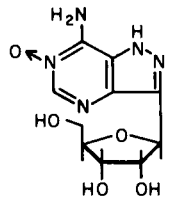



(34)

Structure analysis of 2-methylformycin (34) by X-ray techniques has established the conformation of the C-glycosidic bond as *syn* [150]. In tests for inhibition of leukemia L1210, this compound (34) was more effective than the 1-methyl isomer leading to the speculation [149, 150] that (34), prevented from assuming an *anti* conformation because of steric interactions of the ribosyl moiety and the N-2 methyl group, is an ineffective substrate for adenosine deaminase.

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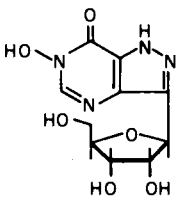


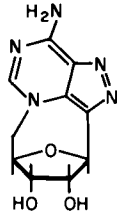

(36)

Long [151] treated formycin with *m*-chloroperbenzoic acid in acetic acid and obtained the corresponding 6-*N*-oxide (35) which, upon reaction with nitrous acid, yielded 6-*N*-hydroxy-3-β-D-ribofuranosyl-7-pyrazolo[4,3-*d*]pyrimidinone (36).

*Anhydroformycins*

In order to prepare formycin derivatives with established, fixed conformations, anhydroformycins (37) and (38) have been synthesized [142]. Reaction of 2′,3′-*O*-isopropylideneformycin with *p*-toluenesulfonyl chloride in pyridine yielded, after deblocking with 90% trifluoroacetic acid, a 3:1 mixture of (37) and (38). Treatment of formycin B (31) with dimethylformide dineopentyl acetal, then with water afforded the *anti* anhydronucleoside of formycin B corresponding to (38) [142].

The *syn* anhydronucleoside (37) is completely resistant to adenosine deaminase (as expected [119]) whereas the *anti* isomer (38) is readily converted by adenosine deaminase to the anhydroformycin B analogue [142].

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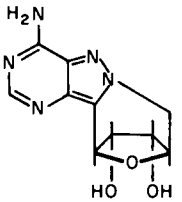


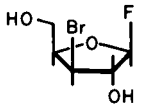

(38)

$N^2$,5′-Anhydroformycin (38) did not inhibit DNA synthesis in murine leukemia L1210 to a significant extent (active at 300 μg/ml) nor did it inhibit the growth of human foreskin cells in culture when added at a concentration of 50 μg/ml [152].

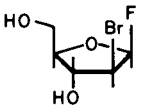
*Ribosyl modified formycins*

A number of derivatives of formycin modified in the ribofuranosyl moiety have been reported. When formycin was allowed to react with 2-acetoxyisobutyryl bromide in acetonitrile followed by acetate hydrolysis using methanolic ammonia, a mixture of bromonucleosides (39) and (40) were obtained [153]. Treatment of this product mixture with sodium

methoxide produced the 2′,3′-epoxy derivative (41). When (39) and (40) were separated and each was hydrogenated over palladium, the corresponding 3′- and 2′-deoxy analogues (42 and 43) were obtained. When the initially formed mixture of 2′(3′)-bromo-3′(2′),5′-acylated formycins (precursors of (39) and (40)) was treated directly with hydrogen over palladium, hydrogenolysis occurred to yield 2′,3′-dideoxyformycin (44) [153]. Finally, when this initial product mixture was treated with chromous acetate and ethylenediamine in ethanol at –78°C, 7-amino-3-(2,3-dideoxy-β-D-glycero-pent-2-enfuranosyl)pyrazolo[4,3-*d*]-pyrimidine (45) was obtained [154].




(39)

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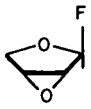


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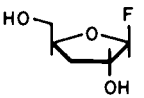


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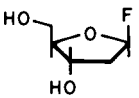


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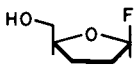


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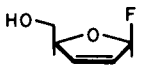



(45)

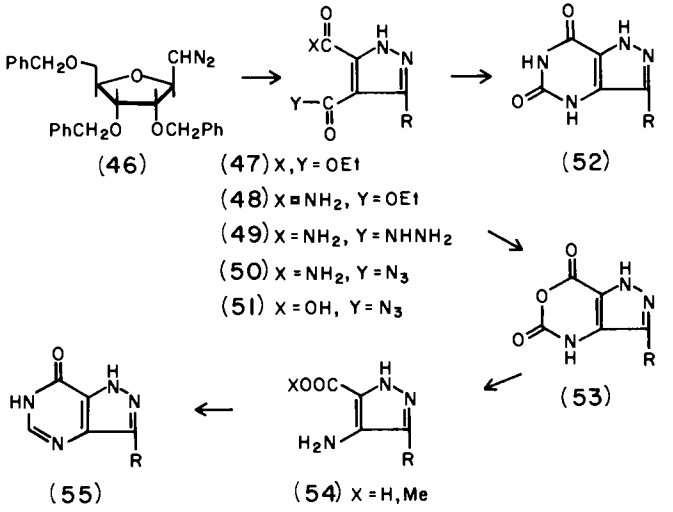
F = 7-amino-3-pyrazolo [4,3-d] pyrimidinyl

## SYNTHESIS OF FORMYCINS

Two research groups–at Stanford Research Institute in the United States [155–157] and that of Farkas and Sorm in Czechoslovakia [158–160]–have developed a synthetic methodology for preparation of the formycins. This methodology, which involves as a key intermediate 2,3,5-tri-*O*-benzyl-β-D-ribofuranosyldiazomethane (46), has been used for the synthesis of formycin B (31) [156] and oxoformycin (32) [159, 160]. Beginning with the diazomethyl carbohydrate derivative (46) [155–161] the nitrogen heterocyclic base was constructed in several steps. 1,3-Dipolar addition of dimethyl acetylenedicarboxylate to (46) produced the dimethyl pyrazoledicarboxylate (47) in good yield. An alternative synthesis of the 3-(ribofuranosyl)pyrazole dicarboxylate (47) involves addition of methyl

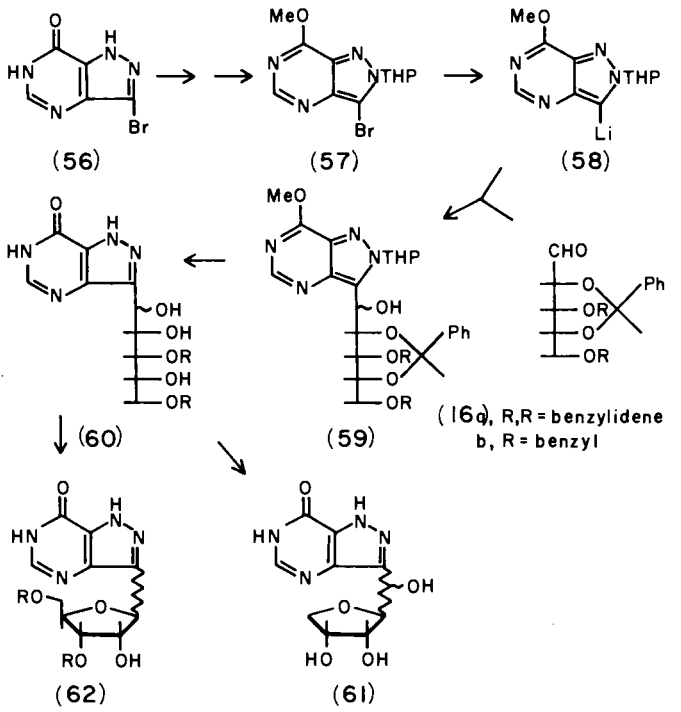
or ethyl diazoacetate to a protected $\beta$-D-ribofuranosyl propiolate at high temperature (50°C) [162]. In this procedure yields are lower (15%) than when a diazomethylcarbohydrate (i.e., (46)) is employed. However, an attractive feature of this latter route is the fact that (47) is available in two steps from ribofuranosyl halides.

Selective aminolysis of (47) [158] yielded (48) which was converted to the hydrazide (49) and then oxidized to the acylazide (50). Curtius rearrangement of (50) proceeded with cyclization yielding the oxoformycin derivative (52) [159, 160]. When, however, (49) was treated with dinitrogen tetroxide in acetic acid–carbon tetrachloride, the resulting acyl azide (51) possessed an adjacent carboxyl group rather than an amide [156]. Curtius rearrangement of (51) produced the anhydride (53) which was hydrolyzed (54, X = H) esterified (54, X = Me) and cyclized with formamide to the formycin B derivative (55) [156].




An alternative synthetic route to formycin has been studied [163]. 3-Bromo-7-pyrazolo[4,3-*d*]pyrimidinone (56) [164] was converted to the protected 3-bromo-7-methoxy-2-tetrahydropyranyl (THP)-derivative (57) which, upon treatment with *n*-butyl lithium, yielded the corresponding organolithium compound (58). Reaction of (58) with either 2,3:3,5-di-*O*-benzylidene-*aldehydo*-D-ribose (16a) or 2,4-*O*-benzylidene-3,5-dibenzyl-*aldehydo*-D-ribose (16b) [165] produced the corresponding C-nucleosides

(59) in good yield (30–50% following chromatography). Treatment of (59) with acid accomplished simultaneous deblocking and carbohydrate ring cyclization. However, the properties of the resulting anhydro product rule out its formulation as the 1′,4′-anhydro compound (62) and are suggestive of the 2,5-anhydro structure (61), analogous to the results obtained by Bobek, Farkas and Sorm [77] during attempted preparation of 6-azapseudouridine (12). Partial deblocking of the differentially protected C-nucleoside (59, R = benzyl) using mild acid catalysis to yield (60, R = benzyl) followed by thermal dehydration produced a mixture of two products exhibiting properties consistent with the anomeric ribofuranosyl C-nucleosides (62, R = benzyl). Definitive characterization of these products and refinement of conditions for blocking group removal and carbohydrate cyclization have not been accomplished.




The β-D-arabinofuranosyl analogue of formycin B was prepared [166] by a sequence analogous to that used earlier for synthesis of formycin B itself (31) [156]. In the process it was discovered, unexpectedly, that

during nitrosation of the blocked β-D-arabinofuranosyl amide–hydrazide corresponding to (49), anomerization occurs to yield the α-anomer [166]. Further, equilibration of the α- and β-anomers occurs during cyclization in boiling formamide (i.e., the transformation analogous to the conversion of (54) to (55)). These results are particularly surprising since anomerization does not occur in the *ribo* series [140, 166].

The lower homologue of oxoformycin (32), 2′,3′-*O*-isopropylidene-DL-erythrofuranosyl-5,7-pyrazolo[4,3-*d*]pyrimidinedione, prepared from 2,3-*O*-isopropylidene-β-DL-erythrofuranosyl diazomethane [155, 156], exhibited activity against lymphoid leukemia L1210 implanted in mice. When this compound was injected intraperitoneally in 9 daily doses of 100 mg/kg, tumour-bearing mice survived nearly twice as long as controls [157]. Synthetic β-D-arabinosyl analogues of oxoformycin [157] and formycin B [166] were inactive against leukemia L1210. The α-anomer of this latter compound exhibited no activity against KB cells [166].

## THE PYRAZOMYCINS (PYRAZOFURINS)

### PYRAZOMYCIN (PYRAZOFURIN)

Pyrazomycin [5-β-D-ribofuranosyl-4-hydroxypyrazole-3-carboxamide, pyrazofurin (63), m.p. 108–113°C, $[\alpha]_D$–47° (c 1.25% in $H_2O$), p$K_a$ 6.7, $\lambda_{max}^{pH7}$ 263 nm, $\lambda_{max}^{pH12}$ 307 nm] was isolated from the culture filtrate of a strain of *Streptomyces candidus* [167–169] and is structurally related to the C-ribosides of the formycin series.

Pyrazomycin (63) prevents proliferation of rhinovirus, vaccinia, herpes simplex and measles virus in cell culture [169] but is inactive against strains of polio, coxsackie A and B, and pseudorabies viruses and shows only limited antifungal activity in vitro. Pyrazomycin is active against vaccinia tail lesions in mice and both the spleen enlargement and polycythemia associated with Friend leukemia virus multiplication in DBA/2 mice are controlled by pyrazomycin given either orally or intraperitoneally [170, 171].

Pyrazomycin inhibits by more than 50% the growth of mammary carcinomas 755 and 115, Gardner lymphosarcoma and X5563 plasma cell myeloma [172, 173]; it was inactive against leukemias L1210, P388, C1498 and P1534 and AKR lymphocytic leukemia. Pyrazomycin did not affect Ehrlich ascites or sarcoma 180 ascites tumours but did increase the

survival time of mice with the ascites form of Walker 256 [173]. Although the Walker 256 carcinosarcoma in rats appeared to be completely supressed by pyrazomycin [173], longer term experiments have shown that, in the presence of pyrazomycin, the onset of tumour growth has a latent period of about 14 days [171]. Pyrazomycin is also inhibitory toward the mammary carcinoma induced in rats by 9,10-dimethyl-1,2-benzanthracene [173].

Plasma levels of pyrazomycin-like activity were monitored in rats which had received a single 10 mg/kg dose [171]. The peak level of activity appeared after 6 to 8 h and substantial levels remained after 48 h. Measurable activity remained for as long as 5 days. This unusually slow rate of drug clearance may explain the ability of pyrazomycin to exert biological effects even when administered only every 3 or 4 days [171].

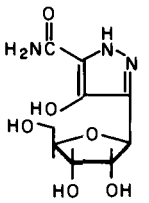
The biological effects of pyrazomycin are reversed specifically by uridine or uridylic acid but not by cytosine or orotidine indicative of a mode of action involving inhibition of orotidylic acid decarboxylase (OAD). It was established that $5 \times 10^{-8}$ M levels of pyrazomycin-5'-phosphate effected a 50% inhibition of OAD from rat liver [169]; pyrazomycin, itself, is inactive. Pyrazomycin 5'-phosphate has been prepared enzymatically and, more recently, by a chemical process which involves prior formation of 2',3'-isopropylidene derivative [171].

It has been proposed [10] that pyrazomycin 5'-phosphate is sufficiently similar to orotidylic acid in chemical and steric characteristics to allow the enzyme OAD to recognize the antibiotic as a substrate. However, X-ray studies of pyrazomycin have shown it to have *syn* conformation [174], a conformation different from that of orotidine. Unfortunately, X-ray data for pyrazomycin 5'-phosphate, which may possess a conformation more similar to that of orotidylic acid, are not available.

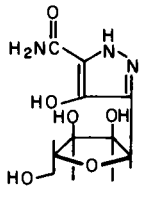
A dose-ranging study using pyrazomycin was performed in eight patients whose diagnoses included renal adenocarcinoma, carcinoma of the breast, acute leukemia, multiple myeloma, carcinoma of the lung and Hodgkin's disease [171]. One patient with Hodgkin's disease (stage IVa, bone) had a three month remission with objective and subjective improvement. A second patient with Hodgkin's disease failed to respond; results with the remaining patients were inconclusive [171]. Clinical evaluations using pyrazomycin are continuing.

## PYRAZOMYCIN B (PYRAZOFURIN B)

Pyrazomycin B (64), the $\alpha$-isomer of pyrazomycin (63), was isolated [174] from the identical strain of *Streptomyces candidus* from which



(63)



(64)

pyrazomycin was obtained and was characterized by spectral, chemical and X-ray data [174, 175]. The status of pyrazomycin B as a natural product is clouded by the recent discovery [171] that aqueous solutions of pyrazomycin undergo facile anomerization above 5°C to produce, at equilibrium, a mixture of pyrazomycin and pyrazomycin B in a ratio of 7:3. This interconversion did not occur in methanol, dimethylformamide, pyridine or dimethyl sulfoxide.

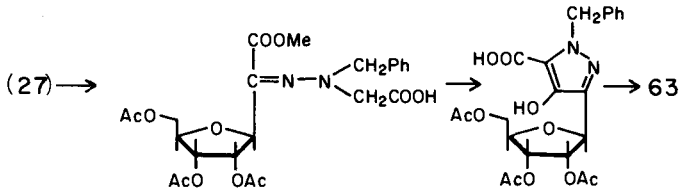
Pyrazomycin B exhibits no antitumour activity and only marginal antiviral activity [171].

## MODIFIED PYRAZOMYCINS

Several acetyl and butyryl derivatives of pyrazomycin have been prepared and tested against Friend leukemia virus in mice and against several tumour systems. In general, these derivatives exhibited biological activities comparable to those of the parent antibiotic; in no case was significantly enhanced activity observed [171].

## SYNTHESIS OF PYRAZOMYCIN

The synthesis of pyrazomycin has been accomplished [176] by a sequence which, in initial steps, is identical to that used for the synthesis of showdomycin (24) [104]. Thus condensation of $\alpha$-keto ester (27) with (1-benzylhydrazino)acetic acid yielded the hydrazone derivative (65). When (65) was heated with sodium acetate in acetic anhydride, cycliza-



(65) (66)

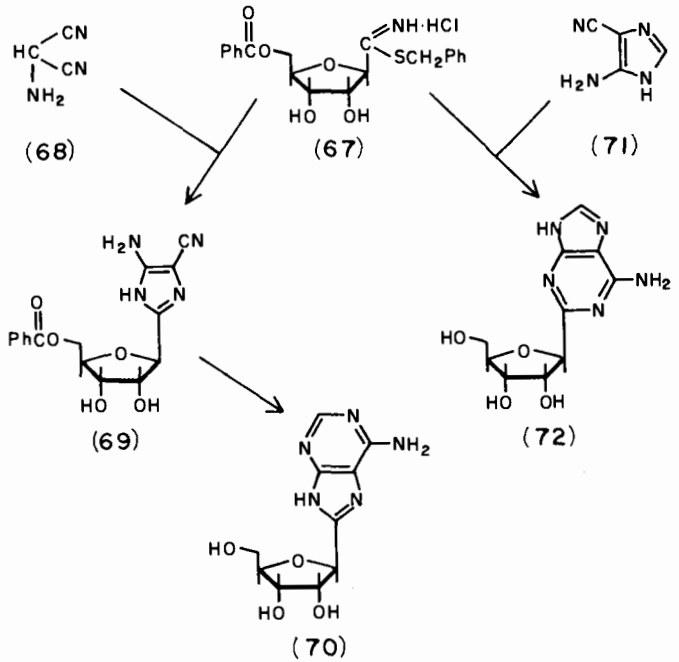
tion occurred to yield the pyrazole-C glycoside (66) which, by conversion of the acid function to an amide and removal of protecting groups, produced pyrazomycin [176].

## SYNTHETIC C-NUCLEOSIDES

During the past several years, a large number of C-nucleosides of various heterocyclic bases have been prepared. It is interesting to note, however, that syntheses of acyclic C-nucleosides were reported as early as 1887 [177]. The following sections comprise a survey of various structure types of synthetic heterocyclic C-nucleosides which have been reported (see also [17, 178]).

### IMIDAZOLE AND PURINE C-NUCLEOSIDES

A number of purines bearing carbohydrate moieties at either C-2 or C-8 have been prepared by procedures involving incorporation of one carbon



of a carbohydrate precursor into the purine skeleton. Thus, condensation of the ribofuranosylthioimidate (67) [179] with aminomalononitrile (68) yielded the imidazole C-nucleoside (69) which, upon condensation with formamidine acetate followed by blocking group hydrolysis, yielded 8-(β-D-ribofuranosyl)adenine (70) [180]. The β configuration for this compound was confirmed by the appearance in the PMR spectrum of a characteristic C-1'H chemical shift and coupling constant, $J_{1',2'}$ [69, 174, 181]. Earlier, Bobek and Farkas [182] prepared 8-(D-ribofuranosyl)adenine by condensation of 4,5,6-triaminopyrimidine with an appropriate carbohydrate derivative. This compound exhibits different optical rotatory and PMR spectral properties and is, in all probability, the α-anomer [180].

Condensation of thioimidate (67) with 4-aminoimidazole-5-nitrile (71) followed by ester hydrolysis using methanolic ammonia yielded 2-β-D-ribofuranosyladenine (72) [180].

*Table 5.3* lists the purine C-nucleosides which have been reported. Very few biological activity data are available for these compounds. El Khadem and Audichya [188] report that 8-(β-D-ribofuranosyl)adenine (70), 8-(β-D-arabinofuranosyl)adenine, 8-(3'-deoxy-α-D-ribofuranosyl)-adenine and 8-(3'-deoxy-β-D-arabinofuranosyl)adenine were tested for

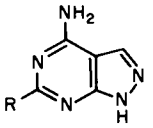
Table 5.3. PURINE C-NUCLEOSIDES AVAILABLE BY SYNTHESIS

| *Purine ring substituents* | | | *References* |
|---|---|---|---|
| *2* | *6* | *8* | |
| β-D-ribofuranosyl | $NH_2$ | H | [180] |
| H | $NH_2$ | β-D-ribofuranosyl- | [180, 182, 183] |
| H | $NH_2$ | α-D-ribofuranosyl- | [182] |
| H | $NH_2$ | 2'-deoxy-β-D-ribofuranosyl- | [184] |
| H | $NH_2$ | 2'-deoxy-α-D-ribofuranosyl- | [184] |
| H | OH | 2'-deoxy-β-D-ribofuranosyl- | [184] |
| H | OH | 2'-deoxy-α-D-ribofuranosyl- | [184] |
| H | SH | 2'-deoxy-3',5'-di-*O*-toluoyl-β-D-ribofuranosyl- | [184] |
| H | SH | 2'-deoxy-3',5'-di-*O*-toluoyl-α-D-ribofuranosyl- | [184] |
| H | $NH_2$ | 3'-deoxy-β-D-ribofuranosyl- | [185, 186] |
| H | $NH_2$ | β-D-arabinofuranosyl- | [187] |
| H | $NH_2$ | 3'-deoxy-α-D-arabinofuranosyl- | [188] |
| H | $NH_2$ | 2'-furyl- | [189] |
| H | $NH_2$ | D-ribotetritol-1-yl- | [189] |
| H | $NH_2$ | D-allopentitol-1-yl- | [189] |

blood schizontocidal antimalarial activity. Each of these compounds was found inactive and devoid of toxicity at doses up to 640 mg/kg [188].

## PYRAZOLO[3,4-*d*]PYRIMIDINE C-NUCLEOSIDES

By a procedure analogous to that used for the preparation of 2-β-D-ribofuranosyladenine (72) [180], five 4-aminopyrazolo [3,4-*d*]-pyrimidine C-nucleosides (73) have been prepared [190]. Interestingly, reaction of the ribofuranosyl thioimidate (67) with 3-aminopyrazole-4-nitrile yielded only the β-isomer (72a) whereas the corresponding thioimidates in the arabinosyl and 2-deoxyribofuranosyl series gave mixtures of α- and β-anomers (73b, c and 73d, e respectively) [190].



(73 a), R = β-D-ribofuranosyl

b, R = β-D-arabinofuranosyl

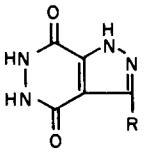
c, R = α-D-arabinofuranosyl

d, R = 2'-deoxy-β-D-ribofuranosyl

e, R = 2'-deoxy-α-D-ribofuranosyl

## PYRAZOLO[3,4-*d*]PYRIDAZINE C-NUCLEOSIDES

During development of procedures leading to the syntheses of formycin B (31) [156] and oxoformycin (32) [159, 160] the pyrazolo[3,4-*d*]-pyridazine C-nucleosides (74a) [159] and (74b) [156] were prepared.

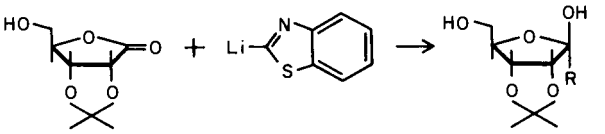


(74 a), R = β-D-ribofuranosyl

b, R = 2',3'-O-isopropylidene-β-DL-erythrofuranosyl

## BENZIMIDAZOLE AND BENZOTHIAZOLE C-NUCLEOSIDES

Ogura and Takahashi [191] treated a series of sugar lactones (e.g., 2,3-*O*-isopropylidene-D-ribono-1,4-lactone, (75)) with various lithiated heterocycles (e.g., 2-lithiobenzothiazole, (76)) and obtained the corresponding C-nucleoside lactols (e.g., (77)). Sodium borohydride reduction produced the corresponding acyclic sugar derivatives. Attempts to eliminate the anomeric (C-1') hydroxyl group were unsuccessful [191]. This result is in sharp contrast to the experience of Asbun and Binkley [66] who successfully utilized this same approach for the preparation of pseudouridine.



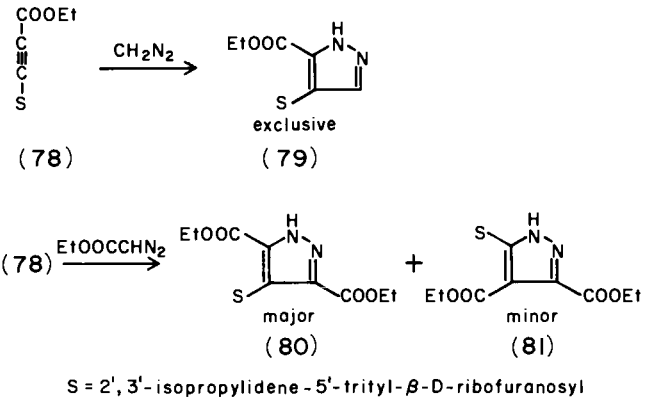

(75) (76) (77)

R = benzothiazol-2-yl

## PYRAZOLE C-NUCLEOSIDES

A number of pyrazole C-nucleosides have been prepared (*Table 5.4*) using a variety of synthetic routes. Pyrazole C-nucleoside precursors for formycin [155, 160] and other related compounds [192, 193] were prepared by 1,3-dipolar addition of acetylene dicarboxylate (or other acetylene [192, 193]) to a diazomethyl carbohydrate derivative. This synthesis leads exclusively to pyrazoles bearing a carbohydrate substituent at C-3.

An isomeric pyrazole C-nucleoside bearing the carbohydrate at C-4 (79) has been prepared [194] by 1,3-dipolar addition of diazomethane to a carbohydrate derivative with an acetylenic function at C-1 (78) [195–202]. When ethyl diazoacetate is used a mixture of pyrazoles, bearing the carbohydrate moieties at C-4 (80) and at C-3 (81) respectively, is formed [75]. By raising the temperature of the 1,3-dipolar addition reaction the regioselectivity of the reaction was decreased and the yield of the minor, 'inverse' addition produce (81) was increased to 15%. A synthesis of pyrazole C-4-nucleosides (e.g., (79), (80)) based on 1,3-dipolar addition of diazoalkanes to olefinic (rather than acetylenic) carbohydrate derivatives has also been reported [203, 204].

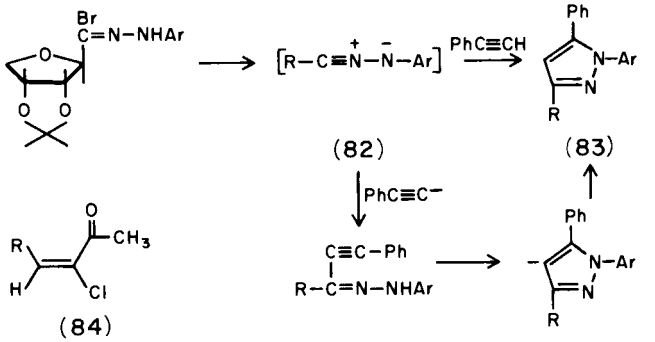



Tronchet, Perret and Jotterand [205–209] have devised a closely related route to pyrazole C-3 nucleosides which involves the intermediacy of a glycosyl-azomethine imine (82) which, when treated with an acetylenic dipolarophile, undergoes two distinct reactions although both

Table 5.4. SYNTHETIC PYRAZOLE C-NUCLEOSIDES[a]

| *Pyrazole ring position (substituent)* | *References* |
|---|---|
| 3(Ph), 4(2',3',4',6'-tetra-O-acetyl-β-D-glucopyranosyl) | [194] |
| 3($CONH_2$), 4(β-D-ribofuranosyl) | [75, 203, 204] |
| 3,5($CONH_2$), 4(β-D-ribofuranosyl) | [203] |
| 3(COOH), 4(β-D-ribofuranosyl) | [203] |
| 3(COOMe), 4(β-D-ribofuranosyl) | [75, 203] |
| 3(COOMe), 4(D-arabino-1',2',3',4'-tetracetoxybutyl), 5(Ph) | [192] |
| 3(β-D-ribofuranosyl), 5(Me) | [210] |
| 2(Me), 3(COOMe), 4(β-D-ribofuranosyl) | [75] |
| 1(*p*-nitrophenyl), 3(β-D-erythrofuranosyl) | [205, 206] |
| 1(*p*-nitrophenyl), 3(β-D-erythrofuranosyl), 5(Ph) | [206, 207] |
| 1(*p*-nitrophenyl), 3(D-arabino-1',2',3',4'-tetrahydroxybutyl) | [208, 209] |
| 3(D-ribo-2',3',4',5'-tetraacetoxybutylcarbonyl), 4,5(COOMe) | [193] |
| 3(D-ribo-2',3',4',5'-tetraacetoxybutylcarbonyl), 5(COOMe) | [193] |
| 3(D-arabino-2',3',4',5'-tetraacetoxybutylcarbonyl), 4,5(COOMe) | [193] |
| 3(D-arabino-2',3',4',5'-tetraacetoxybutylcarbonyl), 5(COOMe) | [193] |

[a] In addition to the compounds listed, numerous carbohydrate-protected C-nucleosides have been reported in refs [155–161, 178] and in those contained in the table. Protected C-nucleosides have been included in the table only when the particular reference reports no deblocked compounds.




ultimately yield the desired product (83). Recently, a new route to pyrazole C-3 nucleosides, involving addition of hydrazine to an $\alpha$-chloro-$\beta$-glucosylvinylketone (i.e., (84)), has been reported [210].

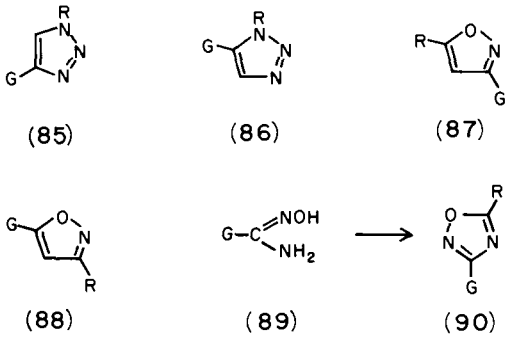
Only two brief reports of biological testing of pyrazole C-nucleoside derivatives are available. Perret (ref. [178] p. 215) reports that 1-*p*-nitrophenyl-3-$\beta$-D-erythrofuranosylpyrazole or 1-*p*-nitrophenyl-3-$\beta$-D-erythrofuranosyl-5-phenylpyrazole at concentrations up to 100 $\mu$g/ml failed to inhibit *Staphylococcus aureus* Oxford or *E. coli* B when these organisms were grown on agar. In liquid suspension culture with either of these nucleosides present at 10 $\mu$g/ml, *S. aureus* grew as well as control cultures. However, under these conditions *E. coli* cultures achieved a turbidity sufficient to decrease light transmission by 50% 9¾ h after inoculation whereas control cultures required 10½ h.

Just and Ramjeesingh [204] tested ethyl 4-(5'-acetyl-2',3'-isopropylidene-$\beta$-D-ribofuranosyl)-2-pyrazoline-6-carboxylate and ethyl-4-(5'-acetyl-$\beta$-D-ribofuranosyl)pyrazole-6-carboxylate as inhibitors of nine viruses (polio, vaccinia, herpes, Coxsackie A21, Semliki Forest, Maryland B influenza, Ann Arbor A influenza, rhino and reo viruses) using agar diffusion and plague reduction assays. No significant virus inhibition or cell toxicity was observed.

## TRIAZOLE, ISOXAZOLE AND OXADIAZOLE C-NUCLEOSIDES

Using synthetic methods analogous to those reported in the previous section, other 5-membered ring C-nucleosides have been prepared. Thus, 1,3-dipolar addition of an azide to an acetylenic derivative of a carbohydrate yields mixtures of 4- and 5-glycosyltriazoles (85) and (86)

[195, 198, 211–213]. When trimethylsilylazide was used [162], a single product (85, 86, R = H) was obtained. Similarly, 1,3-dipolar addition of glycosyl nitrile oxides (prepared by dehydrohalogenation of the corresponding $\alpha$-halo oximes) with acetylenes yielded 3-glycosylisoxazoles (87) [209, 214–218]. Albrecht, Repke and Moffatt [218] prepared 5-ribosylisoxazoles (88) via oxidative cyclization of an $\alpha,\beta$-unsaturated ketoxime. These workers [210] also synthesized 5-substituted-3-$\beta$-D-ribofuranosyl-1,2,4-oxadiazoles (90) by reaction of the amidoxime (89) with acetic anhydride or ethyl acetoacetate.



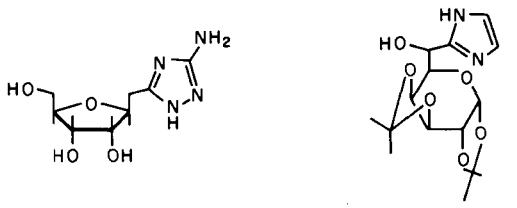
(85) (86) (87)

(88) (89) (90)

G = various carbohydrate moieties

## C-NUCLEOSIDES POSSESSING UNUSUAL CARBOHYDRATE–BASE LINKAGES

C-Nucleosides have been prepared in which the heterocyclic base is linked to the carbohydrate moiety other than at C-1′. Compounds (91) [219] and (92) [220] are representative.



(91) (92)

## SUMMARY

### CHEMISTRY OF C-NUCLEOSIDES

The chemistry of C-nucleosides has become rich with many important accomplishments reported during the past several years. Three general strategies for the preparation of new C-nucleosides are apparent [162]. Numerous new compounds for biological testing have been achieved by modification of natural C-nucleosides. The large body of creative chemistry utilizing the formycins is the outstanding example to date. With but few exceptions [219, 221], synthetic approaches to C-nucleosides have utilized preformed carbohydrates (most often D-ribose) as starting material. Successful syntheses have involved either: (a) direct linkage of preformed base and carbohydrate precursors; or (b) stepwise construction of a heterocyclic base onto a preformed carbohydrate derivative. Another proposed general strategy for C-nucleoside synthesis envisages initial linkage of base and carbohydrate moieties by a bridging sulfur atom with subsequent rearrangement and sulphur extrusion to form the critical carbon–carbon glycosidic linkage. Synthesis of a C-nucleoside by this route has not been accomplished [222, 223].

A consensus has developed (see e.g., [162, 224]) that the most versatile route to C-nucleosides involves functionalization of a carbohydrate derivative at C-1 followed by multi-step construction of a heterocyclic base. A major contribution to the versatility of this route was the recent discovery by Hanessian, Ogawa and Pernet [225–229] that it is possible to condense protected glycosyl halides with stabilized carbanions such as sodio diethylmalonate. This route has now developed to the point that it is difficult to conceive of a potentially interesting C-nucleoside which is not accessible.

The situation with respect to the conceptually more obvious strategy, that involving direct coupling of base and carbohydrate units, is more complex. At the present state of development, this route suffers numerous disadvantages. The principal difficulty is that the most readily accessible metallo derivative of heterocyclic bases, the lithio derivative, undergoes coupling with the $\alpha$-haloether functionality to a very limited extent, if at all [65, 67, 163]. In specific instances, use of the cadmium derivative has proved advantageous [71, 72], however the general solution has been to utilize aldehyde forms of the carbohydrate for the coupling reaction since lithio derivatives of heterocyclic bases are quite reactive

toward the carbonyl group. Unfortunately, while the difficulty with the coupling reaction is overcome, a new problem is created. Achieving cyclization of an acyclic carbohydrate often requires very precise conditions [69] and may occur in an undesirable manner [63, 74, 163], although use of a differentially blocked sugar derivative, e.g., (16b) appears useful in this regard [163].

It seems clear that if this general synthetic route is to compete favourably with other available methods it will be necessary to develop base derivatives which will undergo facile condensation with glycosyl halides. Recent developments in the chemistry of organo lithium cuprates [230] suggest that investigations in this area might be promising.

### C-NUCLEOSIDES IN BIOLOGY AND MEDICINE

The numerous recent research reports included in this review show that interest in C-nucleosides is burgeoning. One C-nucleoside, pyrazomycin (62), is currently in clinical trials as an anticancer agent [171]. The elegant biological experiments using formycin (3) [115, 118] have made great contributions by calling attention to the subtle and critical roles conformation and conformational flexibility of biomolecules play in their normal functioning. Numerous, perhaps a majority, of the synthetic C-nucleosides are only now becoming available for study in biological systems. We can look forward confidently to numerous new and exciting developments as these studies proceed. Now that synthetic methodology is maturing, we can expect many new C-nucleosides, designed to test biological and medicinal hypotheses, to be forthcoming.

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## ADDENDUM

Our literature search has been extended to cover publications appearing up to mid-May 1976. The newly added material has been organized to correspond with the principal subdivision of the review; structure numbers are those used in the main text.

## PSEUDOURIDINE AND RELATED PYRIMIDINE C-NUCLEOSIDES

The occurrence and function of the pseudouridine-containing nucleotide sequence, GT$\psi$C-purine nucleoside, of specific eukaryotic transfer RNAs has been investigated [231]. 4-Thiopseudouridine has been prepared from pseudouridine (1) and shown to inhibit the growth of an *E. coli* auxotroph when cells were grown on (1) but not when uridine (2) was present. It is suggested [232] that the growth inhibitory effect of 4-thiopseudouridine is due to a block in the conversion of pseudouridine to uracil. In a study of over 200 cancer patients with solid tumours, urinary excretion of (1) was uniformly elevated over normal control values regardless of tumour type [233]. These results are consistent with a tumour-associated increase in transfer RNA turnover. Syntheses of two compounds, 5-[(*cis*-3'-hydroxymethyl)cyclopentyl]uracil and its N-1 methyl derivative, viewed as carbocyclic analogues of 2',3'-dideoxypseudouridine have been reported [234].

Pseudoisocytidine (22a) inhibited growth of several mouse and human leukemic cells in culture at concentrations of 0.04 to 3.8 $\mu$g/ml [235]. Similarly (22a) exhibited in vivo activity against various 1-$\beta$-D-arabinofuranosylcytosine-resistant lines of mouse leukemia. These antileukemic activities are blocked by cytidine but not by deoxycytidine or thymidine [235].

## OXAZINOMYCIN (MINIMYCIN)

The biosynthesis of oxazinomycin (23) by *Streptomyces hygroscopicus* has been studied [236] using a variety of radio-labelled precursors and appears to involve a seven or eight carbon sugar as an intermediate. In *S. hygroscopicus*, oxazinomycin and pseudouridine are biosynthesized simultaneously but are not interconvertible despite the close structural relationship and the fact that (23) is readily converted to (1) by ammonia [82].

## SHOWDOMYCIN

Visser [237] has reviewed the chemistry, metabolism and antibacterial effects of showdomycin (24).

## THE FORMYCINS

Yeast phenylalanine transfer RNA, in which adenosine (4) was replaced by formycin (3) at the 3′-terminus, was capable of undergoing all of the individual steps of protein elongation [238] although it exhibited a lag phase and the rate of poly(U)-directed oligophenylalanine tRNA formation was slower than for the unmodified tRNA. This difference is attributed [238] to conformation effects resulting from incorporation of formycin (3) into nucleic acid [239]. The kinetics of conformational changes in phenylalanine tRNA with formycin replacing adenosine at the 3′-terminus have been studied using fluorescence techniques [240]. The fluorescence of formycin has been enhanced by reaction with chloroacetaldehyde yielding a highly fluorescent 1,N-7 etheno-bridged derivative [241].

Formycin acts synergistically with chloramphenicol to control bacterial leaf blight of rice plants [242]. Formycin and fumarate are produced enzymatically from aspartate and formycin B (31) by the *Streptomyces* species MA 406-A-1 [243]. Formycin, formycin B, 1-methylformycin (33), 2-methylformycin (34) and 2′-*O*- and 3′-*O*-methylformycins were tested against vaccinia, herpes simplex and vesicular stomatitis viruses in rabbit kidney cells [244]. Formycin exhibited activity against vesicular stomatitis virus and 2-methylformycin was active against vaccinia virus and nontoxic to the host cells; the rest of the experiment yielded negative results. The antiviral activity for 2-methylformycin and the lack of activity exhibited by 1-methylformycin accords with earlier tests against leukemia L1210 [150]. However, attribution [149, 150] of the differences in biological activity of 1- and 2-methylformycin to the relatively slow rate of deamination of 2-methylformycin by adenosine deaminase is untenable since it has now been shown [244] that whereas 1-methylformycin is deaminated 30 times less rapidly than adenosine (4) and 10 times less rapidly than formycin, 1-methylformycin is essentially unreactive toward deamination by adenosine deaminase.

Further studies on the effects of formycin B (31) on morphogenetic pathways of *D. discoideum* have shown that formycin B interferes competitively with guanosine metabolism [245]. Formycin B inhibits both soluble and chromatin-bound forms of polyadenosine diphosphoribose polymerase isolated from oviduct nuclei of quail [246]. In in vivo studies with mice bearing L5178Y leukemia cells, formycin B was toxic, inhibiting DNA, RNA and protein synthesis. As in the in vitro study, polyadenosine diphosphoribose polymerase of L5178Y leukemia cells was highly sensitive to the presence of formycin B [246].

Oxoformycin (32) exhibits a p$K_a$ of 8.6 identical with that of formycin B. Both the neutral and anionic forms of oxoformycin are strongly fluorescent [247]. A recent X-ray crystallographic study [248] has shown oxoformycin to possess a *syn* conformation about the C-glucosyl bond whereas formycin B possesses *anti* conformation.

## THE PYRAZOMYCINS (PYRAZOFURINS)

A second synthesis of pyrazomycin (63) and pyrazomycin B (64) has been reported [249]. This synthesis involves as a key step the diazotization and concomitant cyclization of 3-oxo-2-(2',3'-*O*-isopropylidene-5'-*O*-*p*-nitrobenzoyl-$\alpha$-D-ribofuranosyl)glutaric acid to form a pyrazole C-nucleoside.

## SYNTHETIC C-NUCLEOSIDES

Synthesis of purine C-nucleosides continues to be of interest [250] and a crystal structure of one of them, 8-(2'-*O*-deoxy-$\beta$-D-ribofuranosyl)hypoxanthine, has been reported [251]. A thiazole C-nucleoside was prepared by treatment of an $\alpha$-haloketose, psicose, with thiorea [252]. Condensations of glucosyl thioformimidates (e.g., 67) with aminocarbethoxypyrazoles allowed preparation of $\alpha$- and $\beta$-anomers of 5-(ribofuranosyl)- and 5-(2'-deoxyribofuranosyl)-7-pyrazolo[4,3-*d*]-pyrimidinones, i.e., isomers and analogues of formycin B (31) [253]. Some new 4-($\beta$-D-ribofuranosyl)pyrazoles have been synthesized [254] by the method reported previously [194]. Isoxazole amino C-nucleosides have been prepared [255].

An important extension of previous work has resulted in the synthesis of 5-thio-8-(D-ribofuranosyl)-3-pyrazolo[1,5-*a*]1,3,5-triazinone which, by desulfurization, will produce an interesting analogue of formycin B [256]. $\beta$-D-Ribofuranosyl-1,4-benzoquinone, synthesized by Kolvada [257], exhibits showdomycin-like antibacterial activity; it exhibits toxicity towards wild-type *E. coli* while mutants deficient in constitutive nucleoside permease are resistant [258]. The $\alpha$ anomer and the isomeric 4-($\beta$-D-ribofuranosyl)-1,2-benzoquinone are inactive.

New results in carbohydrate chemistry which are important to C-nucleoside syntheses continue to appear [256–264]. Tronchet [265] has provided a useful review of the extensive studies of C-nucleoside syntheses undertaken in his laboratory.

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