PROGRESS IN MEDICINAL CHEMISTRY 14

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Progress in
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Preface

There are six reviews in this volume, two on membranes, two on antibiotics and one each on flavonoids and opiates.

The review of Dr A.W. Cuthbert discusses the properties of model membranes and epithelia, particularly in relation to sodium channels and the transfer of ions across cell membranes. This exciting new field involving ionophores is being extensively studied by biological chemists at the present time. The second review by Dr G.J. Moody and Dr J.D.R. Thomas describes some applications of ion-selective electrodes in medicine, an area in which membranes may lead to important advances.

Antibiotics continue to attract attention and the antifungal polyene antibiotics are discussed in Chapter 3 (by Dr S.M. Hammond). Nuclear analogues of the β-lactam antibiotics are considered in Chapter 4 (Dr J.Cs. Jászberényi and Dr E.T. Gunda), structure–activity relationships, which were briefly discussed in Volume 12, now being of great significance for further advances.

Professor P.S.J. Spencer and Dr R.D.E. Sewell discuss in Chapter 5 important changes in brain amines that occur during analgesia with narcotic and non-narcotic opiates and compounds with similar properties. Finally, Dr R.E. Hughes and Dr H.K. Wilson (Chapter 6) expand on the physiological and nutritional significance of flavonoids on the stability and action of ascorbic acid in the tissues.

In conclusion, we wish to thank our authors for their reviews and their patience, owners of the copyright of diagrams for permission to reproduce them, and, by no means least, the publishers for their co-operation and understanding.

February 1977

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G.B. West

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1 Aspects of the Pharmacology of Passive Ion Transfer Across Cell Membranes

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INTRODUCTION

Most writers of reviews, and this one is no exception, find it necessary to introduce their work with a few paragraphs describing the limits within which they intend to contain their discussion. This device allows them to write on those aspects of the title which they find of particular fascination, even though the title at first glance promises greater coverage. The literature on drug effects on membrane permeability to inorganic ions is vast, yet very little is known of the translocation process itself. One aim of this review is to concentrate attention on the transfer process for ions across the membrane.

Passive ion transfer across cell membranes refers to ion movement without the expenditure of metabolic energy by the cell. The driving force for such movements are the existing electrochemical gradients across the membrane, even though these may well have arisen from the prior expenditure of metabolic energy.

A change in membrane permeability to one or more ions is almost the universal way in which a living cell responds to a stimulus, although there are important exceptions with some hormonal stimuli. Pharmacologists are well used to the idea of contracting muscles, conducting nerves and secreting glands and connect these events with a primary change in membrane permeability which leads eventually to the biological response. One has only to pause a moment and realise that many other biological processes are similarly triggered. For example, the response of sensitised mast cells to antigen [1], the response of retinal rods to photons [2], and the fertilisation of ova [3] are all associated with changes in membrane permeability.

The control or modification of membrane permeability by chemical agents is therefore of extreme importance both for understanding the underlying biological processes and for controlling them in abnormal or pathological conditions. The majority of drugs which can be used as tools to investigate membrane permeability are either natural materials or are serendipities from vast screening programmes. This is testimony to our ignorance of the permeability mechanisms in the membrane of cells.

How do ions cross membranes? Perhaps they pass through simply by diffusion, or perhaps they gain access by tiny holes, pores or channels. Furthermore, the pores may be 'gated'; that is, the pores need to be triggered open, as is almost certain to be true for voltage-sensitive channels in excitable membranes [4]. Another possibility is that ions are moved across membranes by special 'carriers' running a sort of shuttle service. The precise details of passive ion transfer are not known for a single biological membrane. For this reason, mechanisms of ion movement across artificial membranes will be considered first in this review. The second section will consider ion movement across biological membranes, but

will deal exclusively with the sodium ion. The reason for this restriction is that more information is available about the translocation of this ion than for others. In addition, sodium ions are moved through some sites which are unassociated with 'receptors'. This last remark requires some further explanation. Drug receptors in membranes are those sites on membrane macromolecules which interact stereospecifically with agonist (or antagonist) drug to initiate (or inhibit) a response. Thus, the receptor is a recognition site which may not necessarily be part of the ionophore, that is, the site responsible for ion translocation [5]. In addition, the recognition site may be coupled to other membrane activities in addition to the ionophore such as adenyl [6] and guanyl [7] cyclases that it becomes unclear whether or not the change in permeability can be dissociated from these events. For example, the response time to muscarinic agonists in smooth muscle cells, monitored as a change in membrane potential, is slow enough to suggest biochemical events following receptor activation may occur [8]. A discussion of voltage-independent and -dependent sodium channels will therefore be included as neither type requires chemical activation. The approach to be followed in the section on biological membranes is rather obvious, namely, what is known of the channels, what is known of the chemical agents which can modify their activities, and can these two be related in a meaningful way.

MODEL MEMBRANES

GENERAL PROPERTIES

There are many types of model membranes from simple partition systems through lipid-impregnated membranes to monolayers and spherulites. Interested readers can find an account of these in an earlier review [9]. The only model membranes from which precise quantitative data can be obtained readily is the lipid bilayer.

To make these membranes, a suitable phospholipid, lipid or mixture of lipids is dissolved in an organic solvent (say *n*-decane); the mixture is gently brushed across a circular orifice in a piece of machined teflon, which itself forms a partition between the two sides of a chamber filled with suitable electrolyte (say 0.1 M NaCl). The diameter of the orifice is usually 1 or 2 mm, and over a few seconds the lipid-containing solution thins down, the excess lipid remaining as a torus around the hole, until a black lipid bilayer remains covering the orifice and separating two salt solutions.

Optical and capacitance measurements indicate that bilayers are extremely thin; in fact, as the name implies, the thickness approximates to two lipid molecules lying end to end. Most bilayers have thicknesses of less than 100 Å, and

most lipids which form bilayers have chain lengths of somewhat less than half this value; for example, distearyl phosphatidylcholine has a chain length of 37 Å. Tilting of the hydrocarbon chains from the normal to the plane of the membrane effectively reduces membrane thickness.

The two lipid monolayers which are opposed to one another to form the bilayer have their polar head groups towards the aqueous phase, while the interior of the membrane consists of hydrocarbon chains held together by short-range Van der Waals attractive forces. It is important to realise that a good deal of solvent (up to 40%) may be retained in the bilayer, although a new technique of bringing two monolayers together allows bilayers without solvent to be studied [10]. By using pure lipids or mixtures of pure lipids, the composition of the bilayer may be known precisely, but in many instances experiments are made with lipid extracts from animal tissues.

The physical properties of bilayers are different from cell membranes in that they have extremely high resistance ($10^9\ \Omega/cm^2$) and usually a low capacitance (typically $0.5\ \mu F/cm^2$). However, when the ionic permeability of bilayers is increased with various agents the resistance falls and the capacitance may increase producing models whose physical characteristics are, superficially at least, similar to biological membranes.

Because lipid bilayers have well-defined physical characteristics which can be measured with ease and accuracy, they have become extremely valuable in increasing our understanding of the ways in which ions may permeate biological membranes.

In the introduction, three ways by which ions may permeate natural membranes were mentioned. These were through channels or pores, using carriers or by diffusion through the substance of the membrane. The evidence that permeation does occur by all three mechanisms in bilayers will now be presented.

CHANNEL (PORE) FORMATION

There is substantial evidence that a number of substances can produce ion-permeable pores in lipid bilayers. These include gramicidin, alamethicin, nystatin and amphotericin B. Their structures are given in *Figure 1.1.* The structures of these substances are different, gramicidin being a linear polypeptide whereas alamethicin is a cyclic polypeptide. Nystatin and amphotericin B belong to the class of polyene antibiotics.

Gramicidin

The first demonstration of pore formation in lipid bilayers came from the innovative experiments of Haydon and Hladky [14–16] with gramicidin. At the time

HCO–L–Val–Gly–L–Ala–D–Leu–L–Ala–D–Val–L–Val–D–Val–
L–Tryp–D–Leu–L–Tryp–D–Leu–L–Tryp–D–Leu–L–Tryp

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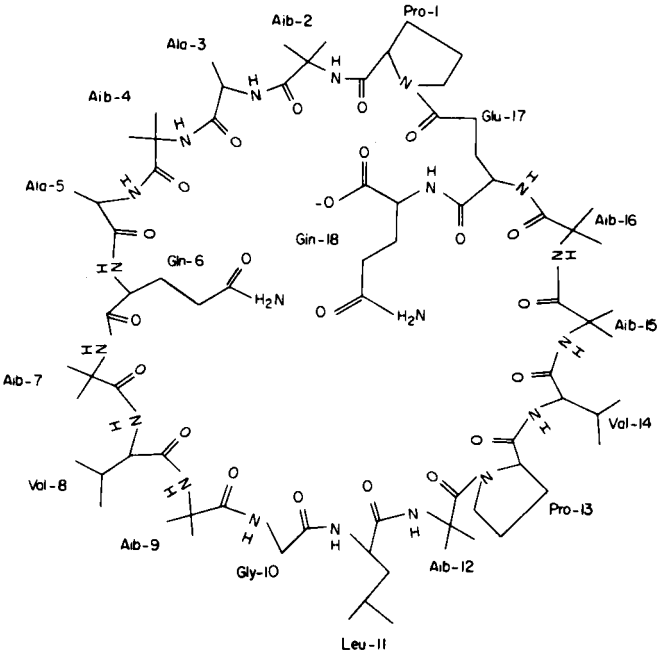

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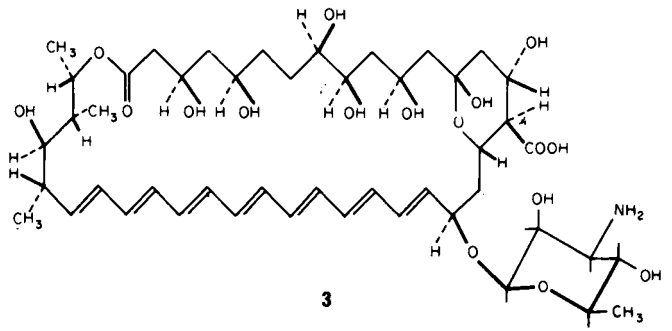

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Figure 1.1. Structure of some channel formers: valine gramicidin A (1), alamethicin (2) and amphotericin B (3)

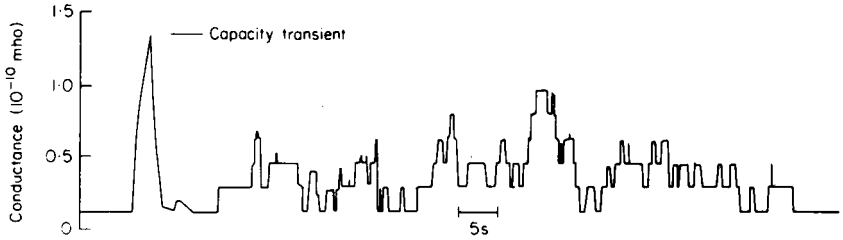


Figure 1.2. Gramicidin conductance transitions with time in a bilayer membrane made from glyceryl mono-oleate and n*-hexadecane. The aqueous phases were 0.5 NaCl, the transmembrane potential was 100 mV, temperature 23°C [207]*

of their studies it was already known that gramicidin increased permeability in mitochondrial membranes [17], the eel electroplax [18] and in red cells [19], but without any idea of mechanism. What Haydon and Hladky did was to measure the current required to maintain a set potential across bilayers in the presence of low concentrations of gramicidin. The current required to maintain the potential is constant unless the resistance of the bilayer changes. They found in the presence of gramicidin that resistance did change but in a rather special way. *Figure 1.2* shows one of their results for a glycerylmonoleate plus hexadecane membrane bathed on both sides with 0.5 M NaCl. Discrete step changes in current were required to clamp the potential, and furthermore the current levels varied by integral multiples while the durations of the step changes were variable. The probability that this type of record resulted from the formation of discrete pores, each with an identical conductance, seemed great.

From analysis of records like the one given in *Figure 1.2*, the properties of the single ionic channels formed by gramicidin were worked out. They were as follows:

1. Channels have an integral conductance and variable duration, both conductance and duration being temperature dependent.
2. The probability of channel termination is constant; that is, termination of a channel is independent of the time it has been conducting.
3. The $I-V$ relationships of single channels are not necessarily linear, channel conductance approaching a maximal value ($5 \times 10^{-11}\ \Omega^{-1}$) at high salt concentration.
4. Channel duration, but not conductance, is dependent upon bilayer thickness [20]. For example, in polyhydroxystearic acid, glycerylmonooleate plus *n*-decane membranes with a hydrocarbon thickness of 64 Å the gramicidin channel has a conductance of $1.7 \times 10^{-11}\ \Omega^{-1}$ and a mean duration of 0.03 s while in a glycerylmonopalmitoleate membrane with a hydrocarbon thickness of 26 Å the mean channel duration is 0.6 s but the conductance is unchanged.

5. The frequency of channel formation rises as the transmembrane potential increases.

To this list might be added the observations that overall mean conductance of bilayers treated with gramicidin is proportional to the concentration of the antibiotic.

Taken altogether, these properties indicate that the discrete integral conductance changes in lipid bilayers treated with gramicidin result from formation of single conducting channels. The magnitude of the single channel conductance indicates that the turnover at each site is greater than could be reasonably expected from carrier oscillations. Added to this, the independence of site conductance with thickness is difficult to incorporate into a carrier model as the diffusional barrier for a carrier would be expected to vary with thickness. The time dependence of the conductance change which occurs when the membrane area is suddenly changed (by bulging the membrane) indicates that the gramicidin complex interacts simultaneously and strongly with both sides of the bilayer.

The ion selectivity of gramicidin channels can be measured either from bi-ionic potentials [21] or from the conductance ratios of single channels [16]. Both approaches give the same selectivity sequence and do not depend either on membrane composition or thickness. The ion sequence is:

$$H^+ > NH_4^+ \geqslant Cs^+ \geqslant Rb^+ > K^+ > Na^+ > Li^+$$

The sequence is dependent on the binding energy of the ions for molecular groups in the channel relative to the free energy of dehydration of the ions. Complete dehydration is not mandatory. The very high permeability to protons suggests that mobility is the most important factor here, which in turn suggests that the gramicidin pore may be lined with water molecules.

What is the structure of the pore? The model proposed by Urry, Goodall, Glickson and Mayers [22] seems to account for the selectivity data in the most satisfactory manner. In this model, the polypeptide chain forms as π^6(L,D) helix with 6.3 residues per turn with a channel diameter of 4 Å. Two such residues placed end to end have a length of 25–30 Å (*Figure 1.3*). One possibility is that pore formation coincides with the apposition of two gramicidin monomers in opposite leaflets of the bilayer by the formation of H-bonds between their inner ends.

Polyenes

More recently, essentially similar findings to those for gramicidin have been reported for the two polyenes nystatin and amphotericin B [23]. It has long

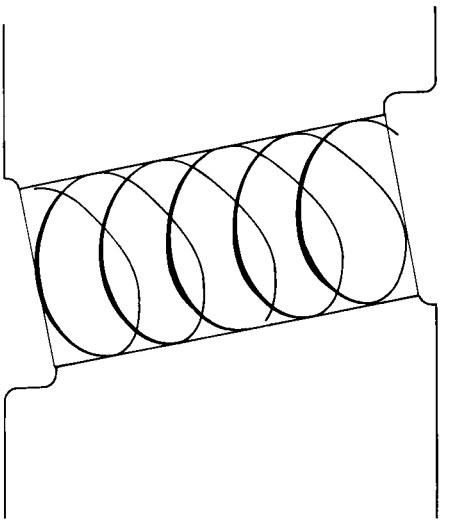


Figure 1.3. Theoretical diagram of a gramicidin channel. Two molecules of gramicidin, hydrogen-bonded at their ends, form the channel. The membrane may be required to 'dimple' if the membrane thickness exceeds the length of the channel (after Urry [22,33])

been known that polyenes interact with cholesterol (and other steroids) present in cell membranes [24–26] and it was found for lipid bilayers that the probability of the appearance of discrete current jumps under a given set of conditions was dependent on the cholesterol : phospholipid ratio of the bilayer. The size of the integral conductance change was independent of the cholesterol concentration. The idea that substances which change membrane permeability may require a particular membrane composition is a recurring theme.

Unit conductances were seen only when the polyenes were added to both sides of the artificial bilayer, reminiscent again of the proposed mechanism for gramicidin pore formation. Many of the properties of single channels formed by the polyenes are similar to those for gramicidin, as for example, complex I/V relations. In this instance, however, the channels are anion selective (for membranes bathed in KCl the transport numbers for K^+ and Cl^- were 0.15 and 0.85, respectively) in contrast to those formed by gramicidin. The structure of the single channels produced by polyenes is unknown, but it seems likely that they consist of polymeric assemblies of polyenes associated with sterols [27].

There seems little doubt therefore that permeant molecules which possess suitable ion binding sites and which are perhaps water-lined provide a means for ion translocation across artificial bilayers.

Alamethicin

In a further example of pore formation in artificial bilayers, the properties of alamethicin appear to be rather more complex than those for gramicidin and polyenes.

The I/V curves for lipid bilayers containing alamethicin are linear over only a small potential range [28], but then conductance increases in proportion to the 6th power of the potential. Apparently, conductance also increases with the 6th power of the salt concentration and the 6th power of the alamethicin concentration.

When alamethicin is examined by the techniques used for observing single channels, complex step-like current fluctuations are seen in which not all the current levels are equally probable and not all the current steps are of equal size [29]. The molecular basis of the events which underlie this behaviour is not fully resolved. The results have been interpreted in ways which suggest that interaction occurs between the individual channels, perhaps by some form of clustering. Presumably then clusters of one size are more probable than others, and also the formation of clusters may alter the conductance of individual channels. Both of these properties would produce the sort of behaviour seen with alamethicin channels.

EIM (excitability-inducing material) and BWSV (black widow spider venom)

Thus far, pore formers with known chemical constitution have been discussed, and clearly these are of greater importance for understanding the microarchitecture of the conducting sites. There are a number of other substances whose structure is unknown, but since they are of biological origin it is sometimes argued that similar substances may be present in animal cell membranes.

EIM, prepared from bacterially desugared egg-white, when incorporated in lipid bilayers, produces membranes which show complex voltage-dependent conductance changes, the so-called bilayer 'action' potentials [30]. Furthermore, discrete conductance fluctuations can be observed with appropriate conditions [31].

BWSV reacts with lipid bilayers, almost irreversibly, to produce cation-selective channels. When the B_5 fraction of the venom (mol.wt. 130 000) is applied to lipid bilayers, conductance increases linearly with time, more or less indefinitely. The discrete step changes in conductance which are responsible for the overall conductance increase have a unit size of 3.6×10^{-10} mho and are unusual in that once formed, the channel appears to be permanently embedded in the membrane. Gangliosides are able to halt the conductance increase by the toxin, probably by binding with it. The treated membranes are selectively cation-permeable

but do not discriminate between Na^+ and K^+, and the channels are reversibly titratable by acid with a pK_a of 5.2 (see also voltage-sensitive sodium channels in excitable membranes).

It is not clear whether the biological effects of the toxin are all sequelae of its effects on membranes. The toxin provokes an increase in miniature end-plate potential frequency at the neuromuscular junction, leading eventually to a depletion of presynaptic vesicles [32]. Increased calcium influx as a result of toxin action on the nerve terminal may be a prime event, but it is difficult to understand the specificity of action of the toxin in the biological situation.

Synthetic pore formers

From a molecular theory of ion-conducting transmembrane channels [33], it was concluded that sequences with the structure *N*-formyl-(L-Ala-L-Ala-Gly)$_n$ would form such channels. A number of such substances have been prepared by using solid-phase techniques for the synthesis of peptides. They are *N*-formyl-(L-Ala-L-Ala-Gly)$_4$OMe, *N*-formyl-L-Ala-Gly-(L-Ala-L-Ala-Gly)$_4$OMe, H^+(L-Ala-L-Ala-Gly)$_5O^-$, H^+(L-Ala-L-Ala-Gly)$_5$OMe, *N*-formyl-(L-Ala-L-Ala-Gly)O^- and *N*-formyl-(L-Ala-L-Ala-Gly)$_5$OMe. All these compounds exhibited channel behaviour when incorporated in lipid bilayers with slight K^+ selectivity [34]. Several different sizes of conductance quanta were measured with the different peptides.

The possibility of designing ion-selective channel formers, particularly when tissue selectivity based upon differing membrane lipid composition can be established, is an exciting challenge for future drug design.

Channel formation in biological membranes

The previous sections on single-channel conductance in lipid bilayers show that pores of molecular dimensions and possessing selectivity can be demonstrated, but can single-channel conductance be demonstrated in biological membranes, or rather, are ions translocated across biological membranes in ways similar to those in lipid bilayers treated with channel-forming substances?

The properties of single channels in some biological membranes has been derived from an analysis of electrical 'noise' [35–37], but more recently single channels have been demonstrated in a biological system [38]. One problem with biological membranes has been to find a system in which there were so few channels that discrete current changes could be monitored. A simple but clever idea was to use a glass micropipette applied to the external surface of a membrane so that a small area of membrane (100 μm^2) could be voltage-clamped. Neher and Sakmann chose the neuromuscular junction for investigation but placed their pipettes outside the end-plate region where the density of cholinergic nicotinic

receptors is low. By including cholinergic agonist drugs in their pipettes, they were able to demonstrate the transient opening of the ionophore associated with the nicotinic receptor in these membranes. Discrete and uniform current jumps corresponding to the insertion of single-channel conductances in the membrane were observed. Their records were not unlike those shown in *Figure 1.2* for the effects of gramicidin on lipid bilayers. The conductance of single channels was approximately 23 pmho, and the duration of the channel opening varied according to the agonist drug used. At a potential of −120 mV and at 8°C, the open time was 43 ms with suberylcholine, 26 ms for acetylcholine and 11 ms for carbachol. This type of result opens a new concept of the meaning of potency in pharmacological terms.

Doubtless, it will not be long before similar demonstrations of unit conductance can be made for other biological systems. There is already some evidence to show that, when subthreshold currents are passed through neural membranes, the potential adjustment proceeds by a series of quantal steps which probably corresponds to the opening of discrete channels [39].

The alternative approach to demonstrations of channel formation in biological membranes is to extract 'channels' from biological materials and then to incorporate these into lipid bilayers where their biological properties can be demonstrated. Little progress has been made so far, probably because biological channels are not easily solubilised and purified, particularly when they are only present in the membrane in small amounts. Furthermore, even when isolated, the native conformation may be irrevocably lost.

Solubilisation of the tetrodotoxin-binding component of neural membranes, believed to be at least part of the voltage-dependent sodium channel, has been achieved. However, the material is so unstable that no biophysical studies have been made with this material [40].

Triton extracts of gastric mucosa contain apparently three materials which can produce channels in lipid bilayers with conductances of 2.5×10^{-10} mho. One material is apparently neutral and cation-selective, another charged, voltage-dependent and anion-selective, whilst the third is non-selective [41]. Material which produces K^+-selective channels in bilayers has been extracted from excitable tissue [42]. The data obtained so far with these natural channel formers are relatively crude compared with the elegant studies with channel-forming antibiotics. Therefore, it is, as yet, unclear whether these materials have definitive roles in biological membrane permeability.

CARRIERS

Lipid-soluble ions

Neither the alkali and alkaline earth metal cations nor their associated anions can permeate lipid bilayers. However, some non-biological ions can do so by virtue of their lipid solubility. Whilst these ions are of no importance in living systems, they may well provide data for understanding the mechanisms by which carrier molecules can translocate ions across cell and other biological membranes.

Tetraphenylboride, dipicrylamine and phenyldicarbaun-decaborane are examples of lipid-soluble anions; tetraphenylarsonium, tetraphenylphosphonium and dimethyldibenzylammonium are lipid-soluble cations [43,44]. Ions listed above can transfer charge across lipid bilayers, zero current potential being 58 mV per decade (that is, a Nernst potential).

Membrane currents with lipid-soluble anions show saturation at high ionic concentrations, and for the anions, at least, the major obstacle to movement is diffusion through the aqueous stationary layers on either side of the lipid bilayer. The large hydrophobic groupings associated with the lipid-soluble ions are able to mask the charge in ways which make it possible for them to penetrate the hydrophobic interior of the bilayers. This immediately suggests a way in which carriers for small ions may operate. What is required are bulky molecules with hydrophobic outer surfaces which may easily partition into cell membranes, yet at the same time contain internal hydrophilic sites which can accommodate a dehydrated cation.

There is evidence that a number of cyclic polypeptides and other compounds can indeed act in this way to promote charge transfer across lipid bilayers. Such materials have been termed ionophores.

Ionophores

Among the compounds considered to act as ion carriers are the following: valinomycin (36, $K > Na$); macrolide actins (monactin, dinactin, trinactin, nonactin) (32, $K > Na$); enniatin (18, $K > Na$); polyethers or crown compounds, 15-crown-5 ($Na > K$), 18-crown-6 ($K > Na$) and 24-crown-8 ($K > Na$). All the compounds listed above are covalently-bonded closed rings and are uncharged. The numbers given above refer to the number of ring atoms, except for the crown compounds where the number of ring atoms and the number of ether oxygens in the ring are given. Also some indication of cation selectivity is included in the list.

In addition, there are numbers of charged carrier molecules such as nigericin ($K > Na$), monensin ($Na > K$) and dianernycin ($Na > K$). These consist of linear

chains of oxygen-containing heterocyclic rings and have a terminal carboxyl group. This group must be deprotonated before ring closure can occur by hydrogen bonding with a suitable OH group at the other end of the chain.

Considerable biological interest has also been aroused by a number of carriers which have some specificity for divalent cations, particularly calcium. Among these are X537A, A23187 and a bromo derivative of the latter.

The structures of some of ion carriers referred to above are shown in *Figure 1.4.* Definitive studies have not been completed on all of these materials and the discussion which follows concentrates on those compounds which have received the fullest investigation. One of these is valinomycin which first received atten-

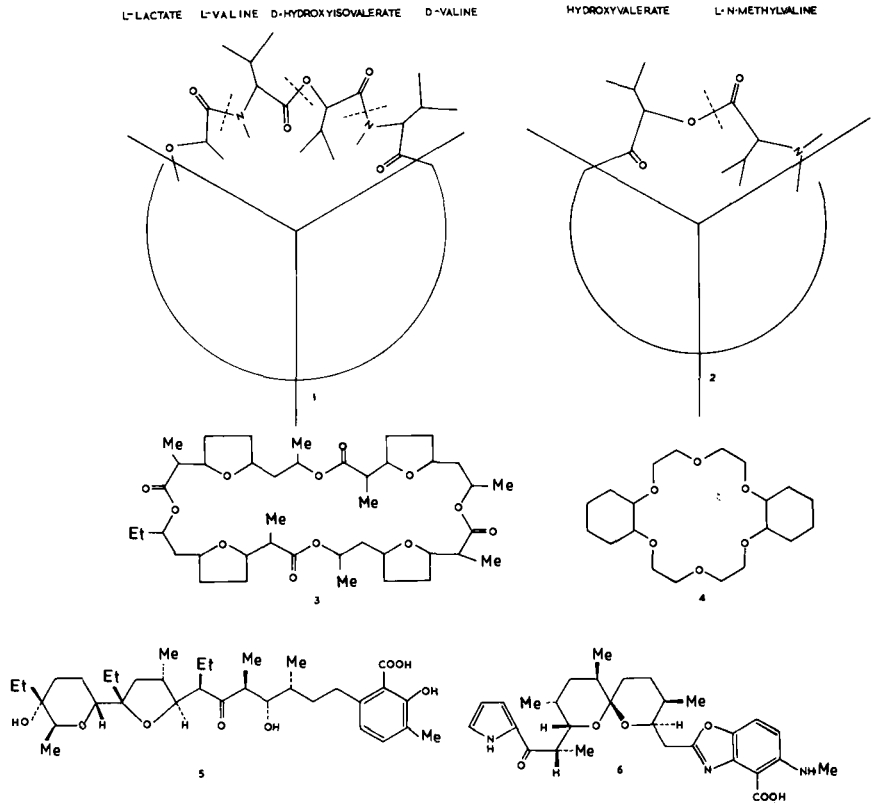


Figure 1.4. Structural formulae of some ionphores: valinomycin (1), enniatin (2), monactin (3), dicyclohexyl-18-crown-6 (4), X537A (5) and A23187 (6). Only partial structures of valinomycin and enniatin are given, the sequences are repeated in the other two sectors. For the calcium ionphores (5 and 6) two molecules of ionphore combine with one of calcium. The methyl ester of A23187 is inactive. The affinity of A23187 for Ca^{2+} *is around* $10^4\ M^{-1}$

tion when it was found that this material could facilitate the transfer of K^+ ions across red cell membranes [45]. Since that time, there have been numerous structural and biophysical studies of this compound. Evidence is presented below that ionophores can sequester ions within their interior, while presenting a hydrophobic exterior to the membrane – the analogy to lipid-soluble ions is then clear. Translocation of ions across membranes by carriers proceeds in a way which allows the ions to run down their electro-chemical gradients, the carrier molecules moving against the ion flux without an ionic load.

What are the characteristics of ion transport by carriers? First, membrane conductivity increases smoothly with ionophore concentration, that is, the discrete changes in membrane conductance seen with channel-forming agents is not observed. Secondly, it should be possible to demonstrate complex formation between the ion and ionophore. Thirdly, ionophores should be able to increase the permeability of both thick and thin lipid membranes, whereas this would not be expected for channel formers as the length of the conducting conformation of the channel would limit membrane thickness. An extreme example of a thick 'membrane' is a simple binary system consisting of an aqueous and a hydrophobic (non-polar) phase. Ionophores should be able to partition ions from an aqueous medium into a non-polar medium, provided a lipid-soluble counter ion is also present so that there is no transfer of charge between the two phases. Fourthly, for a given ionophore, the conductances for different ions should be proportional to the affinities of different ions for the ionophore. That is, the translocation of the complex is largely independent of the ion contained within the complex. Finally, ionophores should have the kinetics of carrier systems.

The kinetics of carrier systems have proved to be rather complex and many of the theoretical predictions are testable only under particularly favourable conditions. Perhaps the most complete treatment has been given by Hladky [46]. His scheme is shown in *Figure 1.5.* As might be expected, the steady-state schemes for lipid-soluble ions and carrier-mediated ion transport are similar. The lower half of the diagram can be considered as the scheme for lipid-soluble ions. Singly primed quantities refer to the left-hand side, and doubly primed the right-hand side, of the membrane. A concentration C'_{is} of a lipid-soluble ion in the well-stirred bulk solution at the left-hand side of the membrane is in a steady-state

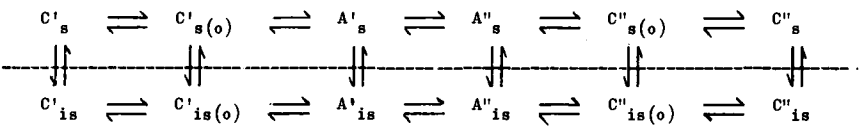


Figure 1.5. Steady-state model for the transport of lipid-soluble ions and for carrier-mediated ion transport [46]. See text for explanation

relationship with the concentration in the unstirred layers associated with the membrane, the concentration at the surface of the membrane being $C'_{is(o)}$. A'_{is} is the concentration of lipid-soluble ion adsorbed to the membrane surface, which is itself in a steady-state relation with the concentration adsorbed on the right-hand side of the membrane. The other quantities on the right-hand side of the membrane have analogous meanings to those on the left-hand side.

In the case of carrier-mediated ion transport, the quantities designated 'is' refer to the complex formed between the carrier and ion while the quantities designated 's' refer to the free carrier. This latter scheme is more complicated as in all phases, except in the interior of the membrane, the ion–carrier complex is in a steady-state relationship with the free carrier. In addition, further complications for carrier transport are introduced when some of the rate constants for the reactions are potential-dependent as well as being concentration-dependent. Introduction of voltage dependency has been necessary to explain the I/V relationships for some carrier systems.

It has been shown for some neutral carriers, for example, valinomycin and the actins, that most of the charge transfer across the membrane occurs by the process *(aqueous ion plus adsorbed carrier)* $\rightleftharpoons$ *(adsorbed complex)* as the diffusion of the complex from the aqueous phase to the membrane cannot be sufficient to supply current [47]. When carriers exist in biological membranes, then this type of reaction probably dominates, as it is unreasonable to expect biological carriers to be lost from the membrane.

Models of carrier-mediated transport are being continuously refined so that the complexity of treatment often obscures the intuitive comprehension of simpler presentations. It is not the purpose of this review to discuss detailed mathematical treatments of carrier transport, but this account would be incomplete without some mention of the differences in carrier-mediated transport in the equilibrium and kinetic domain.

Selectivity in lipid bilayers to different monovalent cations in the presence of carriers can be assessed by measuring the zero current membrane potential, V_0, where

$$V_0 = \frac{RT}{F} \ln \frac{a'_x + (P_x/P_y)a'_y}{a''_x + (P_x/P_y)a''_y}$$

where a' and a'' refer to the activities of two ions, x and y, on the two sides of the membrane [48]. The equation is the Goldman–Hodgkin–Katz equation for anion-impermeable membranes. P_x/P_y is the selectivity of the carrier mechanism for the two ions. The permeability ratios are found to be true constants in those conditions where the rates of loading and unloading of the carriers are fast compared with the rate of translocation of the complex through the lipid interior of

the bilayer. This is the so-called equilibrium domain and the permeability ratios are simply related to the equilibrium chemistry of complex formation. This situation is true for the macrolide actins in asolectin bilayers. For example, the following permeability ratios are obtained for trinactin, $P_{Li}/P_K = 0.00055$, $P_{Na}/P_K = 0.0072$, $P_{Rb}/P_K = 0.47$ [49]. In other situations where the rates of complex formation are slow and comparable with the rate of complex translocation, then the equation given above can still be used but the permeability ratios are no longer constant. This is the so-called kinetic domain, and the permeability ratios are dependent upon kinetic factors and are functions of voltage and membrane composition. This is the situation which exists for nonactin and trinactin in glyceryldioleate bilayers [50]. Readers wishing to discover more details of the kinetics of carrier-mediated ion transport should consult recent reviews [20,51].

It was mentioned previously that it should be possible to demonstrate the existence of complexes between ions and carrier molecules. This has been achieved in a number of instances. Crystal structures of ion complexes with nonactin [52,53], valinomycin [54], monensin [55], nigericin [56], enniatin B [57] and dibenzo-30-crown-10 [58] have been worked out. In addition, there have been structural studies using IR, NMR, ORD and measurements of dipole moments. In general, these have given less clear information about conformation but eventually these approaches may produce information more meaningful in terms of the conformations present in bilayers and in aqueous solution.

What crystallography has shown is that ions, stripped of their hydration shells, are held in a co-ordination sphere supplied by the oxygen atoms of the ionophore, while the external surface of the complex is hydrophobic. While it is uncertain that these structures are maintained in solution, it is highly probable that ions are translocated through lipid membranes complexed in a similar way to those seen in crystal structures.

Apart from a small group of crown compounds, the size of the ring or the number of ring atoms is unrelated to the size of the ions which can form complexes. Thus, when the complex is formed, there are likely to be conformational changes which stabilise the complex. *Figure 1.6* shows the crystal structure of uncomplexed nonactin, in which the torsion angles around A and B are 180° and 70°, respectively, and these become 63° and 150° when the potassium complex is formed. The formation of the K–nonactin complex therefore involves changing 8 torsion angles, but without any change in bond lengths or bond angles. As a consequence of the change in torsion angles, the backbone of nonactin in the complexed state resembles the seam of a tennis ball, in which the ester carbonyls are toward the central cavity. In the K–nonactin complex, the distance from the metal ion to the oxygen atoms, whether ether or carbonyl, does not differ by more than 0.1 Å.

The synthesis of crown compounds by Pedersen [59] allowed compounds

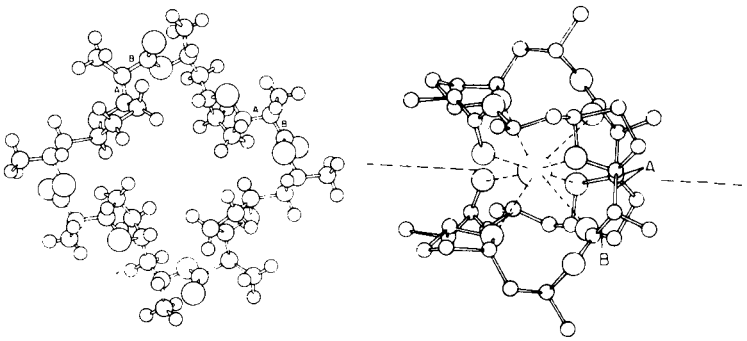


Figure 1.6. The left-hand side of the figure shows the structure of nonactin in the open configuration, with the ester carbonyl oxygens toward the outside of the molecule. On the right-hand side of the diagram the structure of the K–nonactin complex is shown. Conversion of the open to closed configuration occurs by rotation around the bonds A and B. Hydrogen, carbon and oxygen atoms are shown in the left side (circles of increasing size) while on the right side the hydrogen atoms are omitted. The dotted line shows the axis of symmetry of the complex [58]

with varying numbers of co-ordinating atoms (oxygen) to be studied. Dibenzo-30-crown-10 is highly selective for potassium and in the uncomplexed form (*Figure 1.7*) is a linear structure, while when complexed with K^+ the structure is not unlike the K–nonactin complex. The K^+ is held by 10 co-ordinate bonds of approximately equal length, and the conformation change is brought about by changes at four of the C–O bonds. Only a small activation energy is required to bring about this change [60].

For some of the smaller cyclic polyethers, the ring size is an important factor in determining the binding constant for the ion. For example, with dicyclohexyl-18-crown-6, a plot of the binding constant against the ratio of the diameter of the metal ion to the diameter of the hole shows a peak corresponding to the K-complex. Li and Na, which are smaller than K, as well as Rb and Cs, which are larger, have smaller binding constants, with Li and Cs having lower values than Na and Rb, respectively. The peak of the plot occurs at ratio of 0.8–0.9 [61].

Figure 1.7 shows the crystal structure of the silver salt of monesin, an example of one of the monobasic acid carrier molecules. The Ag ion is irregularly co-ordinated by six oxygen atoms. The carboxylate oxygens are hydrogen bonded to OH groups at the other end of the molecule. Divalent ion chelators (A23187 and X537A) also bind head to tail in a manner similar to that of monensin. The stoichiometry for ion complexes of valinomycin, enniatin, nigericin, monensin and the actins (monactin, dinactin, nonactin, trinactin) is 1 : 1, as implied by the crystal structures. This has been confirmed for the

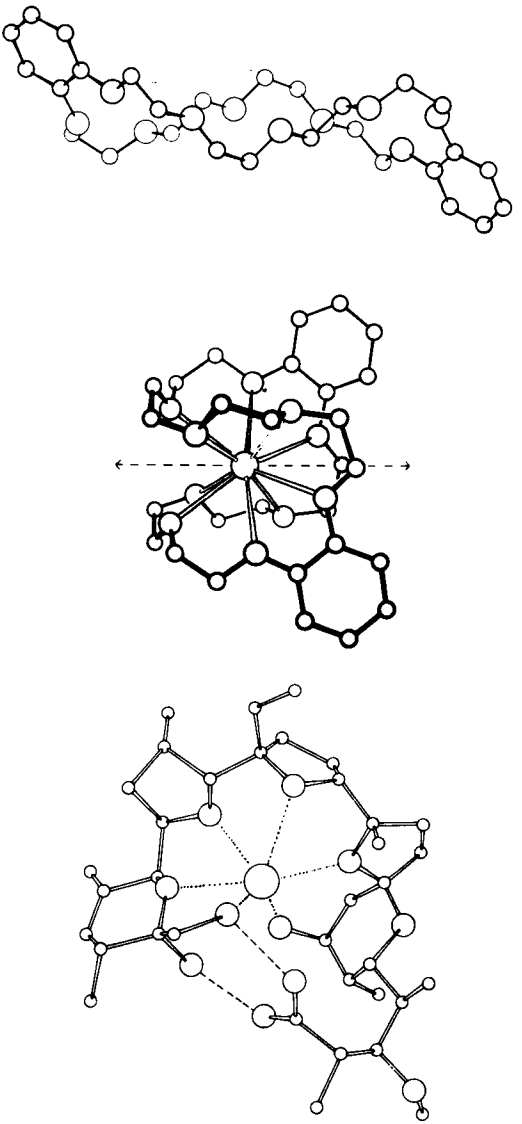


Figure 1.7. Diagram showing the structure of dibenzo-30-crown-10 in the open configuration and as the cationic potassium complex. Small circles represent carbon atoms and large circles oxygen atoms. The dotted line shows the axis of symmetry. The bottom figure illustrates the silver salt of monensin. Carbon and oxygen atoms are represented by small and large circles. Broken lines represent hydrogen bonds [58]

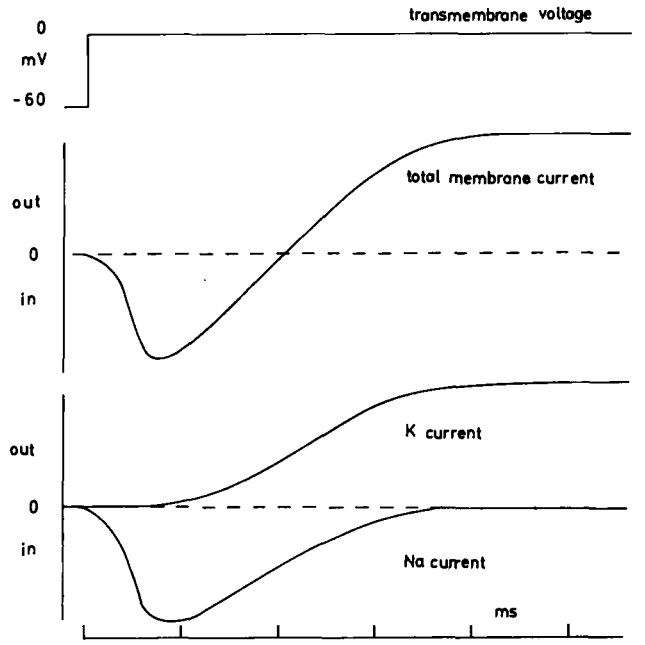


Figure 1.8. Hypothetical diagram illustrating the time course of events in a voltage-clamped excitable membrane. At time zero the membrane potential is changed stepwise from −60 mV to zero, and is held there. In consequence ionic currents flow across the membrane, the net current is first inward, but then reverses to give a maintained outward current. The contributions of sodium and potassium currents which account for the total membrane current are shown

actins, enniatin B and valinomycin by the transfer of metal ions to non-aqueous phases in the presence of lipid-soluble anions [62–64].

Stoichiometries different from 1 : 1 have been found for some ionophores which appear to form sandwiches or stacking structures with ions. For example, dibenzo-18-crown-6 forms 1 : 1 complexes with K^+, 1 : 1 and 2 : 1 complexes with Rb^+, and 2 : 1 and 3 : 2 complexes with Cs^+ [65]. It is unlikely that this type of complex has any relevance for transport studies.

Natural ionophores

After all that has been written in this chapter on ionophores, it is disappointing that as yet there are no examples of natural biological carriers for metal cations. There is, of course, a vast literature on the transfer of ions and solutes across natural membranes, and often the kinetic data are best interpreted in terms of

carrier-mediated transport. Even in such circumstances, ions may pass a good percentage of the membrane thickness by diffusive processes like those of a channel, while a membrane event may be responsible for the final transfer step across the membrane. Turnover numbers for carriers like valinomycin are around 10^4 ions/s while for channels the figure is likely to be two orders higher [66,67]. Ion translocation mechanisms which have features of both carrier-mediated and channel transport probably have turnover numbers in an intermediate range.

Vigorous attempts are now being pursued in many laboratories to extract natural ion carriers from membranes. There is no shortage of criteria upon which such substances can be judged as ionophores. From a pharmacological standpoint, synthetic ionophores may have a place in therapeutics even if the mechanism is novel.

There is one example where materials derived from membranes are claimed to have carrier properties, but real demonstration of their biological function lacks rigour. Tryptic digests of mitochondria contain 9-hydroxy-10-*trans*-12-*cis*-octadecadienoic acid (9HOD) and 13-hydroxy-9-*cis*-11-*trans*-octadecadienoic acid (13HOD). These acids can transfer calcium from an aqueous to toluene–butanol phase and also cause mitochondrial swelling. These compounds have an impressive selectivity for Ca over Mg. 13-HOD also proved to be a potent inhibitor of gramicidin-induced Na^+ translocation in mitochondria and could be the natural ionophore associated with Na^+/H^+ exchange [68].

Finally, and to close the discussion on model membranes, there is an elegant experiment which distinguishes carriers and channels. Bilayers made of glyceryldipalmitate and glyceryldistearate with *n*-decane undergo a transition between 42 and 40°C at which all visible motion ceases. The conductance of such membranes treated with valinomycin also disappears when the temperature is lowered to 40°C, while gramicidin conductance is little changed [69]. The most reasonable explanation (although there are others) is that carrier motion is also inhibited by the viscosity increase while the pores are unaffected.

SODIUM CHANNELS

VOLTAGE-DEPENDENT SODIUM CHANNELS

The conduction of electrical impulses in excitable membranes of nerve and muscle cells depends upon their cable properties and the transient changes in the ionic permeability of the membranes. Our understanding of the permeability changes owes almost all to the classical work of Hodgkin and Huxley [4,70] who applied the voltage-clamp technique to the squid giant axon. They showed that when the membrane was subjected to a step change in membrane potential in

the depolarising direction then the clamp had to supply current to balance ionic currents which flowed as a result of permeability changes triggered by the depolarisation. Immediately after the depolarisation step was applied, the ionic current flowed inwards, then reversed and was maintained in the outward direction for the duration of the pulse. The total membrane current was separable into an inward current carried by Na^+ and an outward current carried by K^+ (*Figure 1.8*). Sodium permeability was rapidly activated but then became inactivated, although the membrane remained depolarised. On the other hand, potassium permeability was activated after a short delay and remained activated for the duration of the depolarising pulse. In squid axon the current was ohmic; that is, there was a linear relation between the current and voltage. Thus at any instant the sodium current was given by

$$I_{Na} = g_{Na}(E - E_{Na})$$

where E is the membrane potential and E_{Na} is the equilibrium potential for sodium. g_{Na} is the sodium conductance and is given by

$$g_{Na} = m^3 h \bar{g}_{Na}$$

where $\bar{g}_{Na}$ is the maximal sodium conductance with all the sodium channels activated, and m^3h represents the fraction of channels which are activated. The terms m and h are dimensionless parameters which are continuous functions of voltage and time. Thus m^3h is the probability that three m 'particles' and one h 'particle' will occupy the correct positions to open the channel.

In other situations the instantaneous sodium currents are non-linear functions of potential and can be more closely approximated by the Goldman equation, as for example in nodes of Ranvier of *Xenopus* [71–74]. In this situation, membrane permeability to sodium is given by

$$P_{Na} = m^2 h \bar{P}_{Na}$$

where $\bar{P}_{Na}$ is the maximal permeability when all sodium channels are activated. The sodium current is given by

$$I_{Na} = P_{Na} F w \frac{C_{Nao} - C_{Nai} \exp w}{1 - \exp w}$$

where $w = EF/RT$ and C_{Nao} and C_{Nai} refer to the sodium concentrations on the inside and outside of the membrane. In yet other situations, for example, myelinated nerves of *Rana pipiens*, sodium currents are again a non-linear func-

tion of voltage and sodium permeability is given by $P_{Na} = m^3 h \bar{P}_{Na}$ [75]. Thus, although the original work on squid axon has been modified and reinterpreted to meet the requirements of other excitable membranes, no alternative hypotheses have displaced the ideas of Hodgkin and Huxley. There can be no doubt that voltage-dependent changes in the permeability properties of membranes are responsible for their excitability. Independent measurements of net sodium influx during the course of a 'membrane' action potential confirms the equivalence with H–H predictions.

Rather recently, it has become possible to measure membrane currents in squid axon in the absence of ionic currents which are thought to represent the movement of mobile charges or dipoles. Part of this current, at least, is thought to refer to the movement of gating particles controlling the voltage-dependent increase in sodium conductance [77,78]. This equivalence is based on the finding that time constants were the same as for the H–H *m*-system, the transition potential (potential at which charges were evenly distributed on the two sides of the membrane) was the same as for the *m*-system, and co-operativity between the charges in groups of three was in agreement with the *m*-system. To abolish ionic currents, all sodium on the outside of the membrane was replaced with Tris, and furthermore a high concentration of tetrodotoxin was added. The inside potassium-containing solution was substituted by CsF. From the aspect of this review, it is of considerable interest that the recognition or selectivity control of the sodium channel (blockable by tetrodotoxin) is separable from the gating function of the channel.

The physical description of sodium currents in excitable membranes, together with evidence cited earlier [39], makes it certain that sodium moves through the membrane by some sort of channel. When the temperature is lowered, nerve membranes show no abrupt changes in ionic conductance as is found in artificial bilayers incorporating carrier substances [69].

In the rest of this section, the properties of sodium channels in excitable membranes will be considered, in particular their selectivity and reactivity to a variety of drugs and chemical agents.

Selectivity

The selectivity of the sodium channels in excitable membranes may well depend on the ability of the ion to interact with fixed anionic or hydrogen bonding sites, together with the requirement to lose some or all of the water from the co-ordination sphere. The free energy of solvation and interaction with anionic sites will increase with ionic radius, but other subtleties of channel architecture may play important roles.

In the squid axon, the selectivity sequence for the sodium channel is Li =

Na > K > Rb > Cs [79]. In this instance, the channel has a small, but finite, permeability to calcium, being one hundredth of that to sodium [80]. In other situations, for example, barnacle muscle [81], the channels associated with the early conductance change admit calcium in preference to sodium. When the calcium concentration of the external solution bathing squid axons is increased, the curve relating sodium conductance to potential is shifted to the right along the voltage axis; that is, greater potential changes are required to produce a given conductance [82]. This effect does not result from blockade of the sodium channels by calcium, but rather calcium adsorbs to the membrane surface so that the field which controls the gating function of the channels is modified.

Two recent papers have highlighted some rather subtle effects on the permeability of sodium channels in excitable membranes caused by changes in the ionic composition of the solution bathing the inside of the membrane. The selectivity ratio P_{Na}/P_K for sodium channels in squid axon fell from 12.8 to 5.7 to 3.5 as internal potassium was reduced from 530 to 180 to 50 mM. These changes were not due to changes in ionic strength or membrane potential [83]. Similarly, in giant axons of *Myxicola,* the P_{Na}/P_K ratio decreased when intracellular K was lowered. Caesium and rubidium were more potent in this respect than potassium, yet neither of these ions are measurably permeant. Thus, channel selectivity appears not only to reflect structural features but also to indicate that the filter has a labile organisation [84].

Hille [85], using frog nodes, showed that the sodium channel was also permeant to some small organic molecules such as guanidinium, hydrazine and hydroxylamine but was impermeable to others such as methylamine. Only the latter is incapable of hydrogen bonding with possible fixed anionic sites in the membrane.

Chemistry of channel blockers

There are a number of substances with well-defined blocking actions on voltage-dependent sodium channels in excitable membranes. Of these the best known is tetrodotoxin (TTX). The characteristics of the blockade by TTX are as follows. The peak transient inward (sodium) current in voltage-clamped membranes is reduced or abolished by TTX while the late steady-state (potassium) current is unaffected [86]. The peak transient current was found later to be blocked by TTX, regardless of the direction of the current and of the ion carrying the current; that is, the specificity of the drug depended on its interaction with the channel [87]. The toxin is effective only when applied to the outside surface; that is, it is ineffective when perfused through isolated giant axons [88].

It is not the intention here to discuss the detailed pharmacology of TTX and like drugs. There have been adequate reviews to which the reader is referred

[89–91]. In this review, emphasis will be placed on chemical aspects of TTX-like molecules.

TTX is derived from the ovary and liver of the Puffer fish or from eggs of the Californian newt, *Taricha.* The structure of TTX is given in *Figure 1.9* [92], where it is shown that the toxin can exist in two cationic forms or as the zwitterion. By examining the blocking effect of the toxin at various pH values, it is concluded that the cations are the active species [93], but whether the activity residues equally in both of these is not known. Desoxytetrodotoxin and tetradonic acid, derivatives of TTX (*Figure 1.9*) appear to have no, or extremely low, activity [94]. Most investigators have found that chemical modification of TTX

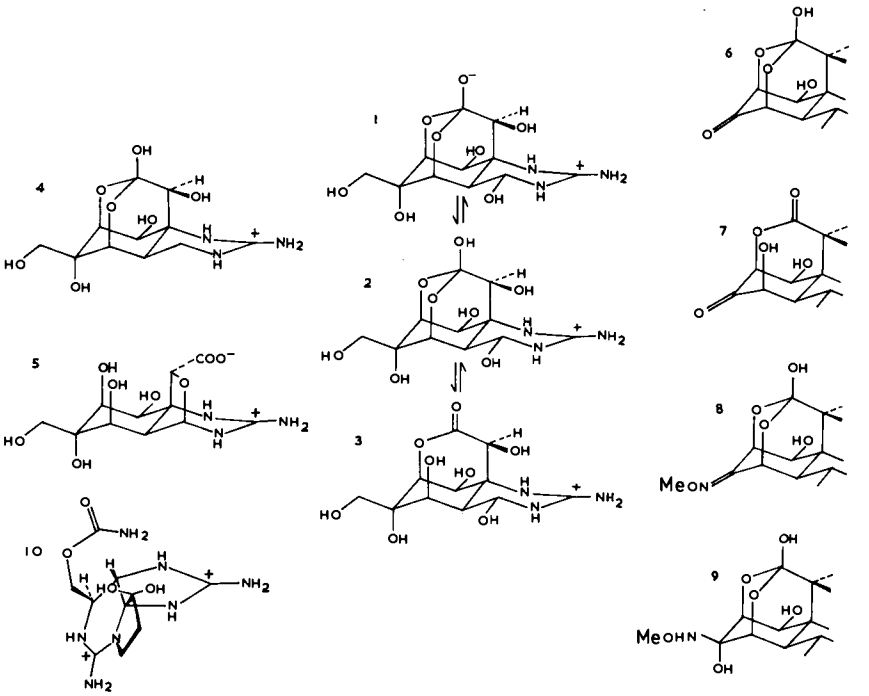


Figure 1.9. Structure of TTX and related compounds. TTX can exist as a zwitterion (1) and in two cationic forms (2, 3). The pK_a *for the dissociation of the 10-hydroxyl is 8.8, but the equilibrium constant between the two cationic forms is unknown. Desoxytetrodotoxin (4) and tetrodonic acid (5) are inactive. The structure of 4 indicates that the highly acidic 4-hydroxy grouping is important for activity as well as the guanidinium cation. Partial structures are shown in 6–9. Formulae 6 and 7 are the probable structures for nor-tetrodotoxin, produced by mild oxidation of TTX. The methoxime (8) (or its tetrahedral intermediate (9)) of nor-TTX has biological activity indicating that the structure of the 'back end' of the molecule also influences biological activity. The structure of saxitoxin is also shown (10)*

leads to a profound loss of activity. Furthermore, compounds like 2-aminobenzimidazole and 2-amino-4,4,6-trimethyl-3,4-dihydropyridimidine (with guanidinium groupings in 5- and 6-membered rings, respectively) have little TTX-like activity [95]. As we have seen, chemical modification in the sensitive 4 position next to the guanidinium leads to a profound loss of activity [94]. Recently, it has been found that mild periodate oxidation allows the 'back end' of the molecule to be modified, away from the guanidinium moiety. When TTX is subjected to mild oxidation, it forms nor-tetrodotoxin (by cleavage of the 6–11 bond) which may exist in hemilactal or lactone forms. nor-TTX has either very low or zero biological activity.

It is difficult to be sure that nor-TTX is completely devoid of biological activity as the presence of a minute proportion of unreated TTX cannot be excluded. When nor-TTX is reacted with methoxyamine hydrochloride, neurone-blocking activity returns. This is considered to be due to the formation of the methoxine of nor-TTX or its tetrahedral intermediate (*Figure 1.9*) [96]. One or both of these substances have blocking activity equivalent to 10–30% of that of TTX. The importance of this finding is that it shows, for the first time, that the end of the molecule away from the guanidinium group is also important for receptor binding and may provide a route for the production of superior labels for the sodium channel.

Saxitoxin (STX), which originates from the dinoflagellate *Gonyaulax catanella,* has the structure shown in *Figure 1.9* [97]. This toxin is probably responsible for outbreaks of shell-fish poisoning which occur when clams are infected with the dinoflagellate. Like TTX, the important grouping for biological activity is believed to be the guanidinium moiety, the rest of the molecule controlling its specificity and affinity. Saxitoxin is more easily removed from tissues by washing than is TTX [98], presumably a reflection of its somewhat lower affinity.

As guanidinium ions can pass through sodium channels in excitable membranes, it is not surprising that both TTX and saxitoxin are considered to be specific for sodium channels by virtue of their charged guanidinium groupings. For this reason, recent results with a further toxin, maculotoxin, which appears to be devoid of guanidinium groups, are particularly fascinating.

Maculotoxin (MTX) is derived from the salivary glands of the octopus *Hepalochlaena maculosa.* It is a cationic neurotoxin, like TTX, and this blocks sodium currents in voltage-clamped squid axons without affecting the potassium current [99]. The chemistry of maculotoxin is incomplete but its probable molecular weight is less than 700. The toxin appears to have one or more basic centres but does not give colour reactions characteristic of guanidinium [100]. While a number of the actions of maculotoxin (block of sodium current, no effect on gating current) are similar to those of TTX, other actions are different. For example, brief repetitive pulses, but not prolonged depolarisation, increase the effective-

ness of MTX, suggesting that the toxin is effective in interacting only with the open channel. Furthermore, MTX alters the sodium conductance kinetics, which is not seen with TTX or STX. The structure of MTX is awaited with interest.

Mechanism of action of channel blockers

Gating currents in sodium channels are measured in the presence of TTX or STX, indicating that the gating portion of the channel is distinct and probably in series with the selectivity filter which allows only sodium (and other similarly-sized ions) through [78,79]. As TTX is effective only from the outside of excitable membranes, the selectivity filter of the channel is probably to the outside of the membrane.

The nature of the selectivity filter in the sodium channel has been investigated principally by Hille [101–105]. He concluded that an ionised acid grouping with a pK_a of around 5 was an essential part of the channel, as g_{Na} fell to low values in acidic solutions. The pH effect was voltage-dependent, the inhibitory effect of protons on P_{Na} being reduced at positive voltages [106]. This result was interpreted as indicating that the ionisable group had a pK_a of 5.4 at zero membrane potential and that the group lies one quarter of the way across the membrane from outside. From studies with organic cation probes, Hille concluded that the selectivity filter was an oxygen-lined slit with dimensions of 3–5 Å, in which the acidic grouping serves to lower the free energy required to dehydrate cations during passage through the selectivity filter. In the more recent of the studies referred to above, a four-barrier model of the sodium channel has been developed, from the Eyring rate theory, to explain deviations from the independence principle. The energy barrier profile of this model is shown in *Figure 1.10.* The height of the major energy barrier (the selectivity filter) determines the current amplitude. It is also suggested from these studies that TTX and STX interact by binding with edges of the channel while the guanidinium group inserts into the slit and interacts with the acid grouping, as originally put forward by Kao and Nishiyama [107]. There are a number of experimental findings which are consistent in general terms with this model. For example, binding of STX to rabbit vagus nerves is inhibited at acid pH in a way which suggests the binding group has a pK_a of 5.9 [108]. Although the blocking effect of protons is pH-dependent, this does not appear to be true for the toxins [109]. Reaction of excitable membranes with carboxyl reagents, such as water-soluble carbodiimides in the presence of a nucleophile, leads to inhibition of toxin binding or a reduction in the sensitivity of the tissue to toxin. In addition, the presence of the toxins can protect against the attack by carboxylic agents [110–111].

Not all excitable membranes are equally susceptible to TTX and STX, even though the action potentials result from transient increases in sodium permea-

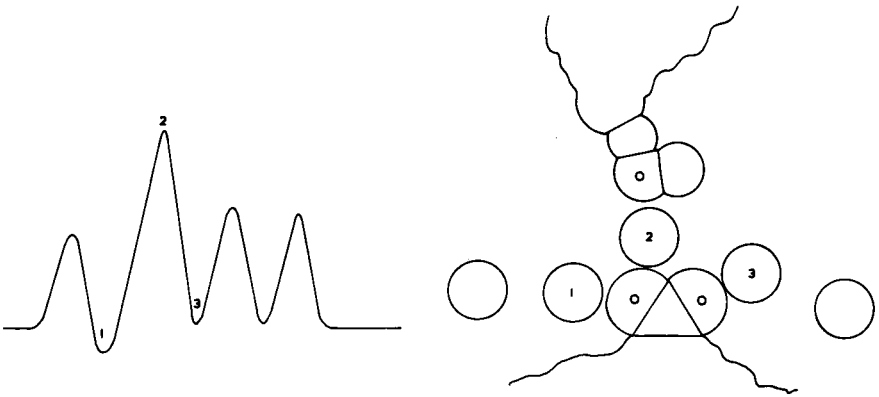


Figure 1.10. Possible way a sodium ion negotiates a channel, according to Hille [101–105]. Sodium ion crosses the membrane from left to right passing four energy barriers. Temporary binding of the ion (to sites 1 and 3) gives the deviations from independence, peak 2 gives rise to selectivity. The hypothetical molecular interpretation (right) shows a water molecule hydrogen-bonded in the channel, and also shows the ion deprived of its hydration shell and partially replaced by the oxygens of the channel (O)

bility [91]. In the newt (*Taricha torosa*) and the puffer fish (*Spheroides maculatus*), the sensitivity to TTX is 30 000 and 1000 less than that expected from values for mammalian C-fibres or squid giant axons [112]. Similarly, effective concentrations in cardiac muscle are around 10^{-6} M, while concentrations 1000 times less than this partially block most mammalian muscle and nerve fibers. It is of considerable interest to know if these variations reflect a different microstructure of the channels or some more fundamental difference in mechanism. Attempts to isolate and purify the sodium channel are at a very elementary stage [40,114,115] and may have to progress much further before this question can be finally answered.

Density of sodium channels

The high specificity of tetrodotoxin and saxitoxin for sodium channels in excitable membranes makes them suitable ligands with which to undertake binding studies on excitable membranes. From such studies, it is expected to demonstrate the presence of a saturable binding component with an affinity for the toxins comparable with the affinity determined indirectly from inhibition studies. This has proved to be so, although some early difficulties were experienced on two fronts. First, it is essential that an accurate specific activity is known for the radioactive toxin. If radioactive impurities (i.e. radioactivity un-

associated with the toxin) are present in the samples, then the values for site density will be incorrect while those for affinity will be correct. This difficulty has largely been overcome by bioassay of the radiolabelled ligands. The second difficulty arises from the presence of the low affinity, high-capacity binding sites in tissues which, in general, add a linear component to a hyperbolic binding curve. Computer fitting of binding curves has become the vogue, and this allows separation of the various binding components and identification of the relevant binding compartment.

The importance of these studies lies in the determination of site density. If site density is known with accuracy, together with a knowledge of the maximal sodium conductance, then the conductance of individual ionic channels can be estimated. The first part of this review was concerned with the alternatives of carrier-mediated, or pore-mediated transfer of ions across artificial membranes. Direct estimates of channel conductances in biological membranes allow tentative conclusions to be drawn concerning mechanisms in biological membranea.

The literature on TTX and STX binding to nerve membranes is now extensive. *Table 1.1* lists a number of biological tissues where the density of sodium channels has been measured with either STX or TTX. The apparent dissociation constant, K, for the toxin is also given.

The affinity of both toxins is a few nanomolar in each example, while the density of sodium channels varies considerably. For nerve fibres, there is a rough relationship between fibre diameter and channel density. It is not possible to make direct measurements of membrane conductance in small fibres, like those of the garfish olfactory nerve. Both in frog muscle and squid axon the channel conductance is a few picomho. This indicates that the turnover numbers at these channels are greater than one could reasonably expect from carrier-mediated transport, yet the turnover is less than at a single gramicidin pore or an ion channel in a muscle end-plate (see p. 7). These latter two, however, lack the ionic

Table 1.1. DENSITY OF SODIUM CHANNELS IN EXCITABLE MEMBRANES

Tissue	*Toxin*	K *(nm)*	*Temperature (°C)*	*No. of channels/ μm^2*	*Estimated conductance per channel (mho)*	*Ref.*
Rabbit vagus nerves	STX	1.8	2–4	110	–	116
Lobster walking leg nerves	STX	8.0	2–4	90	–	116
Garfish olfactory nerves	STX	9.8	2–4	35	–	116
Frog sartorius muscle	TTX	5.0	4	380	10^{-12}	117
Rat diaphragm muscle	TTX	6.1	21	21	10^{-10}	118
Squid axon	TTX	–	ambient	553	2.9×10^{-12}	119

specificity of sodium channels in excitable membranes, so it is probable that sodium ions are forced ro react with the channel during their passage across the membrane with a resultant reduction in site conductance.

In the squid axon, channel density has been calculated from the rate of onset and offset of the effect of TTX on sodium conductance. A value of 522 sites/μm^2 was obtained which is almost identical to that derived from TTX binding [120]. An alternative approach has been to calculate sodium channel density from the maximal size of the gating currents in squid nerve. The result, 483/μm^2, is again close to that obtained by direct measurement [121]. Of further interest are the calculations given by Hodgkin [122] who found that the optimal density of sodium channels in an unmyelinated nerve which are required to give a maximal conductance velocity was around $500/\mu m^2$.

Although understanding of the sodium channel has increased in recent times, there is still much to do before the structure and mechanism are finally clarified. Attempts to solubilise the channel [40] are in their infancy, and efforts to understand its physical [124] and chemical [110,123] properties have yet to be informative about mechanism.

Substances increasing sodium permeability

A number of substances increase the sodium permeability of excitable membranes. Their effects on sodium channels are unclear. For example, when a substance causes a permeability increase which is blocked by TTX, then it may be acting directly on the channel or indirectly on some binding site or receptor adjacent to, or some distance from, the channel. It is not easy to distinguish these possibilities in many instances.

Figure 1.11 gives the chemical structures of the substances to be discussed. Betrachotoxin (BTX) is one of the toxins from the Columbian arrow poison frog (*Phyllobates aurotaenia*). It is the 2,4-dimethylpyrrole-3-carboxylic acid ester of 3α,9α-epoxy-14β, 18β-(epoxyethane-*N*-methylimino)-5β-pregna-7,16-diene-3β,11α,20α-triol [125]. The toxin causes the irreversible depolarisation of a number of excitable structures. The effect is antagonised by low external sodium and by TTX. In the squid axon, it is effective when applied to the inner or the outer surface of the membrane. When nerves are simultaneously exposed to BTX and to TTX, they remain polarised; however, when both toxins are washed away depolarisation ensues, indicating that TTX has not prevented irreversible binding of BTX to its receptor although it has prevented the appearance of its effect. When similar experiments are carried out with BTX and the local anaesthetic procaine, then no depolarisation results when both drugs are removed, perhaps indicating that both BTX and local anaesthetics have a common binding site [126–128].

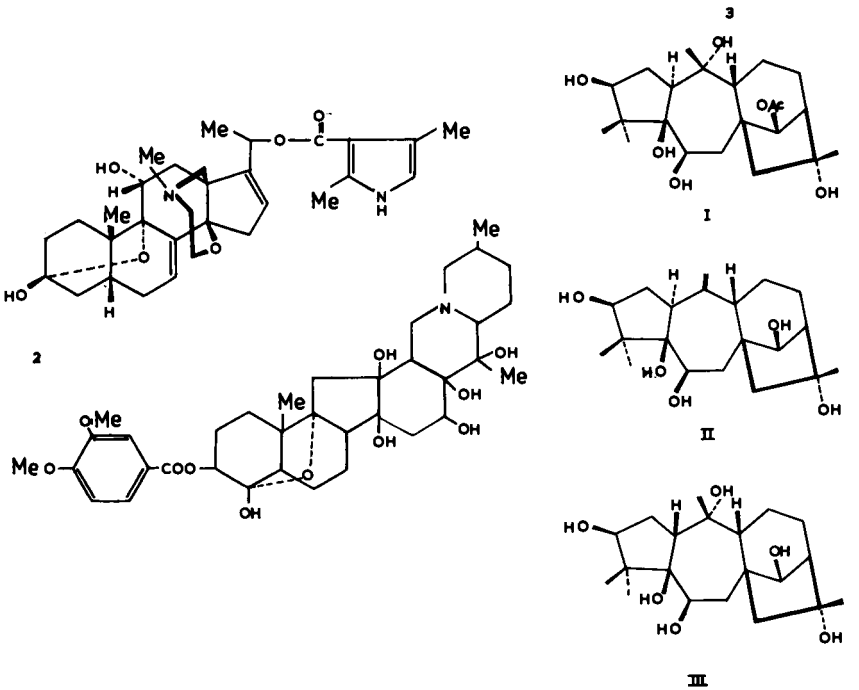


Figure 1.11. Structural formulae of batrachotoxin (1), veratridine (2) and grayanotoxins I, II, III (3)

A group of tetracyclic diterpenoids, the grayanotoxins, extracted from the leaves of plants of the family Ericaceae have similar actions to BTX. The general structure is given in *Figure 1.11* [129–132]. Again, these toxins act when applied to the inner or the outer surface of excitable membranes, are antagonised by low sodium or TTX, but differ from BTX in that they are reversible and can be washed out of the tissue [133].

Much has been written of the veratrine alkaloids derived from plants of the Lilliaceae family. Many early studies were made with natural mixtures of plant alkaloids, and as different alkaloids have differing activities the results are complex and confusing. From studies with a single alkaloid, veratridine [134], it was found with squid axon and frog nodes that the transient increase in sodium permeability was followed by a second, slow-developing permeability increase which did not undergo inactivation. In consequence, many excitable membranes show repetitive depolarisations in response to a single stimulus. The alkaloid is more active when applied to the inside of the membrane, and the cation appears

to be the active species. Reduced sodium and TTX antagonise the effects of veratrine [135].

A species of sea anemone, *Condylactis gigantia*, produces a polypeptide toxin of unknown structure and molecular weight of around 13 000 which affects the kinetics of some sodium channels. In the giant axon of the crayfish (but not of the squid) condylactis toxin prolongs the falling phase of the action potential by delaying the inactivation of the transient sodium current, without pronounced effect on the steady-state potassium current [136].

Local anaesthetics

No account of sodium channels in excitable membranes would be complete without some mention of local anaesthetic drugs. Some new aspects of local anaesthetic action are included, particularly since they infringe on the mechanisms of sodium channels in excitable membranes.

The earlier work on local anaesthetics can be summarised as follows. First, effective concentrations of local anaesthetics are in the millimolar range and when compared with toxins like TTX (which are effective at a few nanomolar) indicates that local anaesthetics act rather non-specifically to perturb the membrane rather than at receptors. Secondly, the variety of structures which exert local anaesthetic action are legion. It was shown first for squid axons that local anaesthetics inhibit both the transient (Na) and late steady-state currents (K) in voltage-clamped axons [137,138]. Some local anaesthetics affect the peak transient-current kinetics, and alter the time course of the gating currents [78]. However, lidocaine has no effect on TTX binding by rabbit vagus nerve, perhaps indicating the independence of the selectivity filter and the gating mechanism of a channel [139].

Initially, there was much confusion in deciding whether the protonated or uncharged form of the tertiary amine anaesthetic was the active moiety. There is now general agreement that the quaterised form is most active and that it is effective when applied to the inner surface of the membrane. This latter requirement was the cause of the original confusion since the drugs need to be in the uncharged form to penetrate the nerve membrane. Intracellular perfusion of giant axons with quaternary compounds with local anaesthetic activity has confirmed these mechanisms [140–142]. It is desirable, of course, for local anaesthetics for clinical use, to have pK_a values around 8.0.

Barbiturates can also act as local anaesthetics and can exist as uncharged or anionic species. For these substances, both penetration and effectiveness depend upon the uncharged material gaining access to the inner membrane surface [143].

The question of what local anaesthetics do to membranes to cause disfunction

has received considerable attention, and the reader is recommended to examine the recent paper by Lee [144]. Not only does the paper provide a fine summary of previous work in this area, but also puts forward a new hypothesis. The evidence against local anaesthetics acting on membrane receptors or by affecting surface charge, causing membrane expansion, altering membrane viscosity, or by perturbing protein structure is neatly summarised. Inspiration is taken from the finding that a number of membrane macromolecules depend for their integrity upon the presence of a lipid annulus, perhaps containing selected lipids, to support the structure. In some instances, the annulus appears to be in a crystalline or gel phase, while in others it is fluidised. The evidence is presented to suggest that relaxation of the sodium channel is prevented by a crystalline annulus which, after fluidisation by incorporation of local anaesthetic molecules, allows relaxation with an increase in height of the energy barriers for ion translocation. The hypothesis has a timely appeal, but one cannot help feeling concerned that local anaesthetics might be expected to reduce TTX binding in this model.

SODIUM CHANNELS IN EPITHELIA

Epithelia not only cover and line the tissues of the body but they also form barriers across which substances move. This movement may be passive or there may be active transport of solutes across epithelia in a way which regulates the contents of the compartments they bound. The list of actively transporting epithelia is large and includes epithelia found in kidney, alimentary tract, duct and gland systems, cornea, extraembryonic membranes, choroid plexus, bladder, genital tract and genital organs. Epithelial membranes are more difficult to study than those of most cells because in general, the tissue is asymmetric, that is, the properties of one face differ from those of the opposite face. Thus, one studies systems with at least two membranes in series with one another.

Ion movements across epithelia can occur through the cells (transcellular route) or between the cells (extracellular route or 'shunt' pathway).

Epithelia like those of the small intestine, proximal renal tubule, and gall bladder are characterised by low or negligible transepithelial potential, low transepithelial resistance, and high hydraulic conductivity, and the shunt conductance is a large fraction of the total transepithelial conductance. These epithelia are able to transport large volumes of isotonic fluid.

At the other extreme, some amphibian epithelia, such as frog abdominal skin and toad urinary bladder, behave as 'tight' epithelia. These are characterised by a high transepithelial potential difference and resistance, and very low hydraulic conductivity, and the shunt conductance is only a small fraction of the total conductance.

Between these two extreme types, there is an intermediate group of epithelia,

with intermediate biophysical properties. Examples are the epithelia of the kidney distal tubule, colon and salivary ducts.

The differences between the various types of epithelia do not seem to depend on the properties of the cell membranes themselves but rather on the variation in the properties of the tight junctions which join the epithelial cells together at their apical borders. When these tight junctions are not highly resistive, then the transepithelial potential is literally short-circuited through the tight junction.

Not all transporting epithelia transport sodium, but when they do there appear to be two general mechanisms. In tight epithelia, sodium enters through the apical surface of the cell passively by passing down either an electrical, chemical or electrochemical gradient. It is removed from the cell by an active process using sodium pumps located in the baso-lateral membranes. By this means, the sodium enters the lateral spaces between the cells and so has been transported across the whole epithelial structure. Under open-circuit conditions, the electrical potential developed across the epithelium serves to drive an accompanying anion, usually chloride, passively across the tissue (*Figure 1.12*). Since the tissues have a low hydraulic permeability there is water flow accompanying solute transport, the driving force being created by the osmotic gradient.

Transport mechanisms in leaky epithelia are less well understood. For example, the proximal tubule and the gall bladder can transport large volumes of isotonic sodium chloride.

A popular model since 1967 has been that of Diamond and Bossert [145]. According to this model, sodium enters the cell and is pumped into the interspace at its apical end by sodium pumps particularly concentrated at the apical ends of the baso-lateral membranes. A local hypertonicity is then created at the blind end of the interspace so that water moving through the cell osmotically increases the volume and pushes the fluid out of the interspace as isotonic fluid. In this way, a standing gradient is set up causing isotonic fluid flow. However, it is now known that in leaky epithelia the tight junctions are permeable to ions and probably to water molecules too. Also, there is no histochemical evidence for a concentration of pump sites at the apical end of the interspaces.

Various modifications of this model have been produced to take account of the properties of the tight junction and of the distribution of pumping sites. For example, a model for isotonic fluid absorption in the proximal tubule of *Necturus* has been worked out as a function of measured transport parameters [146]. In this model, sodium is transported through the cells and pumped into the whole length of the intercellular spaces. The consequences of this on electrical, chemical and osmotic gradients for solutes and water moving through the membranes and through tight junctions is considered. In this model, the emergent concentration of solute differs from exact isotonicity but by an amount small enough to be experimentally indistinguishable from isotonic transport (*Figure 1.12*).

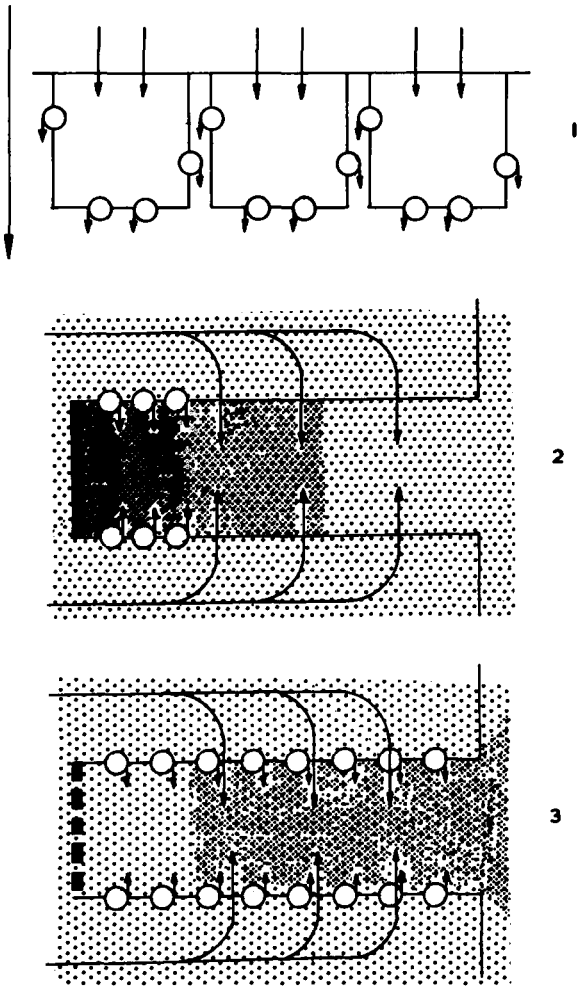


Figure 1.12. Models of epithelial transport. The first model is of sodium transport through a tight epithelium. Sodium (short straight arrows) enters the apical side of the cells down an electrochemical gradient. Sodium pumps (circles with short arrows) in the basolateral membranes expel sodium into the serosal solution. Chloride ions (large straight arrow) move passively through the tissue down the electrical gradient created by active sodium transport. Models 2 and 3 illustrate how solute and water transport may be coupled to give isotonic transport. Sodium which has entered the cells is pumped into the intercellular spaces to give a hypertonic solution which moves water osmotically (curved arrows) into the intercellular spaces. In model 2 [145] the tight junctions are impermeable and the solute pumps are confined to the apical end of the intercellular spaces. The transported fluid is diluted in its passage along the intercellular space and is isotonic when it enters the serosal solution. Model 3 [146] allows a permeable tight junction and has solute pumps along the whole of the lateral space. The transported fluid enters the serosal solution somewhat hypertonic to the mucosal solution. The density of dots suggests the solute concentration

Occurrence of sodium channels in epithelia

It is important to realise that sodium channels in epithelia are different from those in excitable membranes. One clear piece of pharmacological evidence for this point of view is that epithelial sodium channels are relatively unaffected by TTX in high concentrations [147,148]. Furthermore, it is likely that there is more than one type of sodium channel in epithelia. For example, there are a number of pyrazine and pteridine compounds which block sodium transport by blocking sodium entry into the cells of the distal kidney tubule but do not block sodium entry into cells of the proximal tubules.

From the point of view taken in this review, only those epithelia having sodium channels which can be blocked by defined chemical compounds, particularly the pyrazine carboxamides, will be discussed.

As a general rule, channels blocked by the pyrazine compounds are found only in 'tight' but not 'leaky' epithelia. Thus pyrazine carboxamides block sodium entry into the cells of the kidney distal tubule [149], salivary duct [150], colon [151] and epididymis [152], but not into the cells of the small intestine of kidney proximal tubule. Sodium channels sensitive to pyrazine carboxamides are not confined either to epithelia or to mammalian cells. For example, sodium entry into the cells of frog skin epithelium [153], toad urinary bladder [154], chicken coprodeum, fish gills of some species [155], body wall of the leech [155] and crustacean gills [155] are also sensitive. In addition, there are reports that sodium entry sites in ova [3] and erythrocytes [156] are also susceptible to the effects of the pyrazine derivatives.

The wide distribution of sensitive tissues suggests that this type of channel is of considerable evolutionary antiquity as are the voltage-sensitive sodium channels of excitable membranes. Furthermore, throughout the remaining sections of this review, it will become clear that there are some, at least superficial, similarities between sodium channels in excitable membranes and those in epithelia.

Chemistry and mechanism of action of channel blockers

As kidney tubules are target tissues for drugs which block sodium channels in epithelia, a number of useful drugs have emerged from large screening programmes for diuretic compounds. Clinically, diuretics are used to mobilise oedema fluid. Commonly, oedema is a result of congestive heart failure in which glomerular filtration falls, urine output falls, but tubular reabsorption does not decrease to the same extent. Consequently, excess solute is retained in the body together with an iso-osmotic amount of water to form oedema fluid.

Initially, screening tests for putative diuretics are generally carried out on rats. There are two types of tests which are of interest here. First, using saline-

loaded rats, the composition and volume of the urine collected following administration of test compounds is compared with that produced in controls [157]. The results indicate whether or not a substance is natriuretic and some clue as to the site of action may be gleaned from the composition, osmolarity and pH of the urine. Second, adrenalectomised saline-loaded rats, pretreated with deoxycorticosterone acetate, are used. The ratio of urinary Na to urinary K is small in such animals because steroid-dependent sodium reabsorption in the distal tubule is maximised, with a consequent increase in potassium loss from the same segment. Drugs which block sodium reabsorption in the distal tubule cause a rise in the urinary Na/K ratio compared with that of controls [158]. Two sorts of action result in failure to reabsorb sodium in the distal segment of the nephron. Aldosterone antagonists (such as spironolactone) interfere with the action of aldosterone (or other mineralo-corticoids) by competing for binding sites on the cytosolic binding proteins. Consequently, DNA-dependent RNA synthesis is inhibited so that aldosterone-induced proteins are no longer available to stimulate sodium transport. Diuretic substances of this type are not discussed here.

Alternatively, sodium entry through the luminal faces of the cells of the distal tubule may be blocked. Specific blockade of these channels by diuretic substances are now considered with particular reference to their structure–activity relations and mechanisms of action. The following classes of substances have been shown to have blocking activity at these sites: *N*-amidinopyrazine carboxamides, aminopteridines, aminopyrimidines, triazines, and azidopyrimidines, the first two of these groups being the most important.

N-*Amidinopyrazine carboxamides.* The synthesis and biological activity of a great number of compounds of this type were described in a series of papers between 1965 and 1968 [159–162]. The first of these described the diuretic activity in adrenalectomised DOCA-treated rats of a series of *N*-amidino-3-amino-6-halopyrazine carboxamides. In the second paper, it was discovered that the introduction of a 5-amino group into the pyrazine ring increased the diuretic potency. One of the compounds, amiloride (*N*-amidino-3,5-diamino-6-chloropyrazine carboxamide (*Figure 1.13*)) later became widely used in clinical practice, particularly in combination with other diuretics. Its use in combination with diuretics acting more proximally in the nephron is related to the prevention of potassium loss which results when an excess sodium load is delivered to the distal tubule. Thus, amiloride is a potassium-sparing diuretic [163].

All the pyrazine compounds were tested for their ability to increase the urinary Na/K ratio in DOCA-treated adrenalectomised rats. Compounds which produced a 50% reversal of the DOCA effect at a dose of less than 10 μg per rat were given a score of 4. Scores of 3 were achieved with a dose of 10–50 μg, 2 with a dose of 50–100 μg, 1 with a dose of 100–800 μg and ± with a dose of more than 800 μg, the scores given in *Table 1.2* being derived in this way.

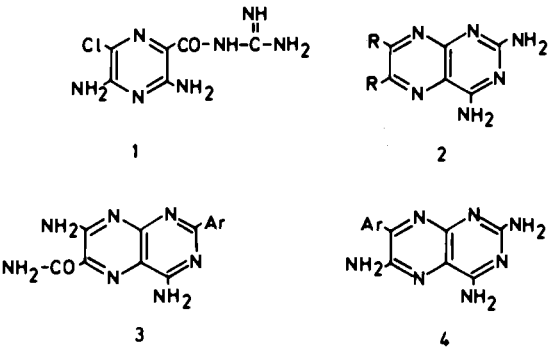
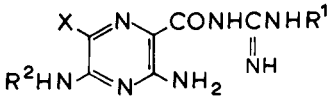


Figure 1.13. Strucrural formulae of amiloride (N-amidino-3,5-diamino-6-chloropyrazine-2-carboxamide) (1), 2,4-diamino-6,7-dialkylpteridine (2), 2-aryl-4,7-diaminopteridine-6-carboxamide (3) and 2,4,7-triamino-6-arylpteridine (4). Triamterene is 2,4,7-triamino-6-phenylpteridine

Table 1.2. STRUCTURE–ACTIVITY RELATIONSHIPS OF PYRAZINE CARBOXAMIDES



R^1	R^2	X	*Score*
H	H	Cl	4
H	H	Br	3
H	H	I	3
H	H	H	1
H	H	MeS	±
CH_2Ph	H	Cl	4
$CH_2C_6H_4$-4-Cl	H	Cl	4
$CH_2 \cdot C_{10}H_7$	H	Cl	3
$CH_2 \cdot CH_2 \cdot OH$	H	Cl	4
H	Et	Cl	3
H	C_3H_7	Cl	3
H	CH_2-CH $\diagdown\diagup$ CH_2	Cl	3
H	$CH_2 \cdot CH_2 \cdot OH$	Cl	1
H	$CH_2 \cdot CH=CH_2$	Cl	4
H	CH_2Ph	Cl	1

It was found that a chloro substituent in the 6 position produced greater activity than other halogens, other groupings or no substituent at all. By contrast, the guanidinium grouping appeared to be a permissive part of the molecule, even large substituents producing little or no reduction in activity. The 5-amino substituent in the pyrazine ring was necessary for high activity, but substitution on this grouping generally reduced activity.

The effect of these compounds on DOCA-treated rats is probably not a very sensitive measure of activity. For example, the benzyl derivative of amiloride shows at least a 10-fold increase in affinity for the channels compared with amiloride when measured in a suitable *in vitro* system [148].

Aminopteridines. A variety of aminopteridines were investigated using a simple rat diuretic assay [164]. The three main groups of compounds examined were 2,4-diamino-6,7-dialkylpteridines, 2-aryl-4,7-diaminopteridine carboxamides, and 2,4,7-triamino-6-arylpteridines (*Figure 1.13*).

Of 22 compounds of the first group, only one, 2,4-diamino-6,7-dimethylpteridine, was active in the sodium-deficient (high endogenous aldosterone) diuretic test. The phenyl, *p*-totyl and *m*-totyl derivatives of the second group were active diuretics in sodium-deficient rats, but unlike 2,4-diamino-6,7-dimethylpteridine and amiloride, these compounds were also kaliuretic as well as natriuretic. In this group, some substitution on the carboxamide nitrogen was possible without destroying activity.

Of the triaminopteridines, the 6-phenyl compound (triamterene) proved to be active in the saline-loaded and sodium-depleted rat. Triamterene also reduced K excretion like amiloride and proved to be active also in dog and in man [166]. Only modest changes in the phenyl ring are possible if activity is to be retained. However, the phenyl group can be replaced with non-basic heterocycles (e.g. furyl), or some alkyl radicals (methyl, ethyl and *n*-butyl) without losing activity.

It is interesting to consider the activity of the four triamterene isomers. The 6-phenyl and 7-phenyl isomers are equally active in sodium-deficient rats, but the 7-phenyl isomer has only poor activity in the saline-loaded rat. The 4-phenyl isomer is without appreciable activity, while the 2-phenyl isomer has modest activity but, as with 2-aryl-4,7-diaminopteridine-6-carboxamides, it is kaliuretic as well as natriuretic.

Aminopyrimidines. A number of substituted pyrimidines had a short-lived clinical usefulness and were developed because of their partial structural resemblance to the xanthines. Aminometradine and amisometradine (*Figure 1.14*) produce a sodium chloride diuresis together with a small potassium loss, but without changing the glomerular filtration rate. Presumably, therefore, they act upon reabsorptive processes in the tubule. However, since they are ineffective in patients with sodium depletion, it is unlikely they act on the distal tubule to block sodium channels [167,168]. Some substituted isocytosines are also

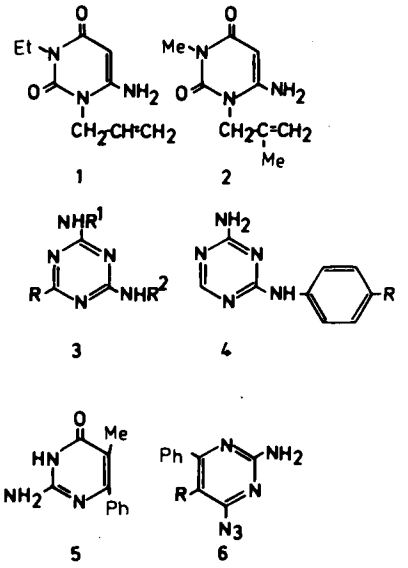


Figure 1.14. Structural formulae of some diuretic substances: aminometradine (1), amisometradine (2), melamine (R = NH_2, $R^1 = R^2 = H$) (3), formoguanamine (R = $R^1 = R^2 = H$) (3), diacetylformoguanamine (R = H, $R^1 = R^2 = Ac$) (3), amanozine (R = H) (4), chlorazanil (R = Cl) (4), 6-methyl-5-phenylisocytosine (5) and azidopyrimidines (R = $CH_2 \cdot CH_2 \cdot OEt$ or $CH_2 \cdot C{\equiv}CH$) (6)

diuretic, for example, 6-methyl-5-phenylisocytosine [169] is active in animals and man (*Figure 1.14*).

Triazines. A number of triazines such as melamine, formoguanamine and its diacetyl derivative (*Figure 1.14*) were found to be diuretic in rats and man [170, 171] but their clinical use was limited by the tendency to produce crystalluria. Some *N*-substituted 2,4-diamino-*s*-triazines (e.g. amanozine and chlorazanil) were particularly potent in rats [172,173], dogs [174] and man [175]. Activity was lost in disubstituted amino derivatives. The effects of DOCA on urinary Na and K are reversed by chlorazanil, suggesting that the diuretic exerts at least part of its effect on the renal distal tubule [176].

Azidopyrimidines. The diuretic activity of a group of 2-amino-4-azido-6-phenylpyrimidines was found to depend upon the nature of the substituent in the 5 position [177,178]. For example, the 2-ethoxyethyl derivative had general diuretic properties like the thiazides but in addition it was more potent than spironolactone as a mineralocorticoid antagonist. The 2-propynyl derivative (*Figure 1.14*) was also a potent antimineralocorticoid but showed no general diuretic activity. Both compounds were potent antagonists of ADH.

In the foregoing paragraphs, the activity of a number of classes of diuretic substances which block sodium transport in the kidney distal tubule has been discussed. It remains to examine these structures to assess whether or not a particular chemical grouping is responsible for this activity. Mechanisms of action of diuretics are difficult to examine on the kidney itself but it is fortunate that there are a number of model epithelia which behave very similarly to the distal tubule. For example, amphibian skins and bladders [179,180] actively transport sodium from the mucosal to the serosal side by an active transport process. These epithelia respond to aldosterone with an increase in sodium transport [181] and furthermore, under appropriate conditions they can be made to acidify the mucosal solution [182]. Amphibian epithelia, therefore, provide ideal systems for examining the actions of diuretics which act on the distal tubule.

In amphibian epithelia (skins and bladders), amiloride blocks sodium transport when applied to the mucosal face [153,154,183–185]. The essential part of the molecule is the guanidine moiety, as the methyl ester of 3,5-diamino-6-chloropyrazine carboxylic acid is without activity [154]. Furthermore, it is necessary for the guanidine group to be protonated for blocking activity [148, 186–188]. The pK_a of amiloride is 8.7 and is therefore largely protonated in the conditions existing in the distal tubule. In the absence of calcium ions (and in the presence of a calcium chelator), amiloride fails to block sodium entry [189] although it apparently still binds to the receptor [190].

Substitution on the guanidinium group may increase affinity (for example, as in benzylamiloride), provided the substituent is not limiting with respect to the conformation of the guanidinium grouping and, of course, provided the substituent does not limit access to the receptor [148].

In triamterene, the 2-amino group with the two adjacent ring nitrogens is isoteric with the guanidinium group in amiloride, and it seems likely that this region becomes protonated first at suitable pH values. The pK_a of triamterene is 6.2 [148,165]. The triamterene isomer with the phenyl group in the 7 position is almost equiactive to triamterene, while the 4-phenyl compound is inactive, perhaps as the phenyl causes steric hindrance in this latter position. Although the 2-phenyl compound has diuretic activity, it is also kaliuretic which suggests that it does not act on the distal tubule. This is in accord with the above hypothesis as the 2-amino grouping is absent. As 2,4-diamino-6,7-dimethylpteridine shows triamterene-like activity, it appears that the phenyl group in either position 6 or 7 is important to increase affinity by providing a large group for hydrophobic bonding.

The affinity of the sodium channels in amphibian epithelia for amiloride and triamterene-like drugs is dependent on the ambient sodium concentration [191, 192]. At 111 mM NaCl, the affinity is around $10^7\ M^{-1}$ for amiloride and around $10^6\ M^{-1}$ for triamterene.

No reports have appeared of the effects of triazines and pyrimidine diuretics on amphibian epithelia but since they block transport in the distal tubule it is likely that they are effective in the model systems. Like amiloride, they contain the guanidinium isotere which may be relevant to their activity. It is worthwhile to remember that toxins like TTX and STX (which block sodium channels in excitable membranes) also depend on guanidinium groupings for their activity.

The sodium channels in epithelia are also blocked by protons, as in excitable membranes, the channel titrating with a pK_a of around 5 [148]. Also agents attacking carboxyl (or phosphate) groups can modify the properties of the channel [193], again analogous to the situation in excitable membranes. Thus, both types of sodium channel have a wide distribution in nature and there are enough similarities to suggest that they may have a common ancestry.

Density of sodium channels in epithelia

The high affinity of amiloride for sodium channels in epithelia, particularly at low sodium concentration, has made possible the development of methods for measuring the density of sodium channels in the mucosal surface [194–196]. All the experiments were carried out under conditions where the mucosal surface was bathed in a low sodium solution (1.1 mM). It was found that the affinities of amiloride (and triamterene) determined from binding studies were in good agreement with the affinities measured when these agents were used as inhibitors of transport under the same conditions [190,196].

Unlike sodium channels found in excitable membranes, the density of the channels was found to vary as a result of various biophysical perturbations. As the methodology allowed the simultaneous measurement of amiloride binding to the saturable, high-affinity component of the membranes and the level of transepithelial sodium transport which could be maintained by the tissue, a number of important correlations were made.

For example, when tissues were deprived of sodium for several days the site density increased 3-fold from around 250/μm^2, but more importantly there was a proportional increase in the level of transport which was maintained by the epithelium [197] (*Figure 1.15*). This effect seems an important homeostatic device possessed by the cell to deal with exposures to divergent sodium concentrations. Furthermore, the result indicates that the entry step, rather than the exit step from the epithelium which requires sodium pumping, is the rate determinant of the level of transport. The time course of the effect of sodium deprivation may mean that it is dependent on the *de novo* synthesis of new membrane permeases. However, increasing the membrane potential across the mucosal face of the cells causes an immediate appearance of new channels, while reducing the potential does the converse [198], indicating that there are binding sites (and

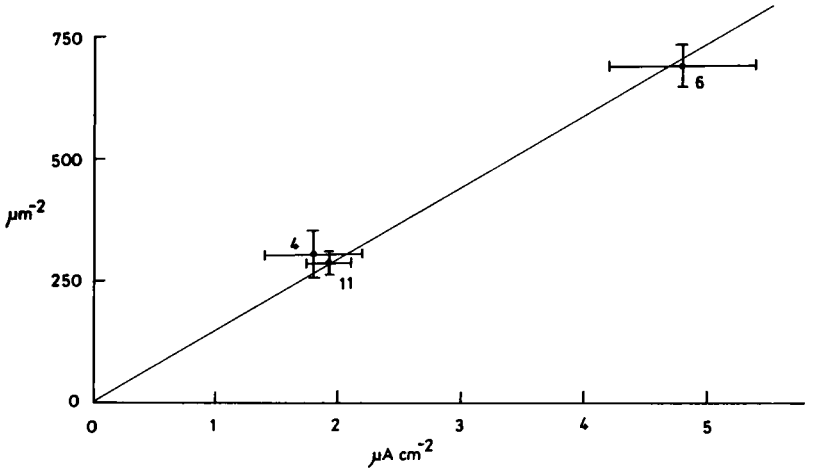


Figure 1.15. Density of sodium channels in frog skin epithelium versus the level of sodium transport which can be maintained by the tissue. Sodium channel density was determined by measuring [^{14}C]amiloride binding. Sodium transport was measured as the clamping current required to maintain the transepithelial potential at zero, with a low sodium solution (1.1 mM) bathing the mucosal surface. The six epithelia represented by the extreme right-hand point had been deprived of sodium for 1 week. The values for 11 untreated skins and for 4 sham-treated skins are shown at the right. Mean values and standard errors are illustrated. Redrawn from Cuthbert and Shum [197]

presumably channels) which can either be occluded or exposed by alterations in membrane potential. Increases in intracellular sodium concentration at constant extracellular concentration cause a reduction in the density of available channels [199], as does an increase extracellular concentration of sodium at near constant intracellular levels. Both of these effects are immediate [193,200] and again are probably expressions of the ability of these membranes to control the density of sites, more likely for cellular homeostasis rather than for the benefit of the organism as a whole.

Biophysical studies on these membranes have indicated that the channels open only intermittently, and from the size of the individual current pulses it seems unlikely that the ions cross the membrane by a simple carrier mechanism [207]. Fluxes of several million ions/s are calculated to occur, whereas the upper limit for simple carriers is more likely to be around 10^4 ions/s.

The effects of hormones

Sodium transport across amphibian epithelia (and kidney epithelia, too) can be increased by two hormones, antidiuretic hormone and aldosterone. Antidiuretic hormone (ADH) also increases water permeability in these situations and this is,

of course, its most important effect on the mammalian kidney.

The effects of hormones therefore, override the cellular homeostatic mechanisms for the sake of homeostasis for the organism as a whole. In the previous section, it was noted that the entry step for sodium was the rate determinant of the level of sodium transport which can be maintained by the epithelia. There is a large amount of evidence, which cannot be reviewed here, to suggest that both of the hormones which increase sodium transport do so by modifying the entry step; that is, they increase sodium permeability.

Binding studies with amiloride have given results which support this viewpoint but, more importantly, have also given some clues as to mechanism. Antidiuretic hormone was found to have no effect on channel density but to increase the mean current flowing in each channel while at the same time the affinity of amiloride was reduced [190,196,201]. Consideration of these results led to the postulate that ADH increases the fraction of channels which are conducting at any instant or alternatively the open time of the channels is increased by ADH [202]. That ADH (or rather its second messenger) is an allosteric activator of the channels, altering the proportion of open-to-closed channels still remains the best working hypothesis which takes account of the existing data.

The effects of aldosterone are different. First, the onset of its effect takes at least one hour, while ADH takes only a few minutes. Aldosterone causes an increase in the density of channels [196,201,203] with no change in apparent affinity to amiloride. Thus, as far as the probe molecule is concerned, the new channels appear to be identical to the pre-existing channels. The effect of aldosterone on channel density is blocked by inhibitors of transcription and translation [196], indicating that *de novo* protein synthesis is involved in the effect. Whether the new protein is actually new channel or whether the new protein can expose pre-existing but occluded sites in the membrane has not been answered.

The turnover of sodium channels in epithelial membranes is slow. After inhibition of protein synthesis, the half-time for the disappearance of the channels is approximately 60 h [204].

The complexity of the changes of amiloride-binding site density, and hence presumably of sodium channels, which can take place in these epithelia are only just beginning to be understood. It is not surprising, therefore, that the ideal diuretic has not been found, for any perturbation at one part of the nephron is likely to affect the ambient sodium concentration at a distal part. The ensuing adaptive changes, plus the influence of hormones, are such as to produce complex patterns of excretion.

Sodium channels in tight junctions

The characteristics of 'leaky'epithelia were given on p. 33. The properties of these epithelia depend on the nature of their tight junctions. For example, in

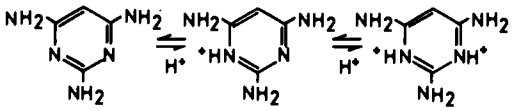


Figure 1.16. Structural formulae for triaminopyrimidine. The acid dissociation constants are 6.74 and 1.31

Necturus gall bladder, 95% of the ionic conductance pathway follows an extracellular route [205].

Triaminopyrimidine (TAP, *Figure 1.16*) has been found to decrease the sodium conductance of several leaky epithelia including frog, rabbit and *Necturus* gall bladders, rabbit and bullfrog intestine and bullfrog choroid plexus [206]. The impressive feature of this result is that there is no effect on chloride conductance, which may mean there are even separate ion channels in tight junctions. The magnitude of the sodium effect is large enough to indicate that the major part is on tight junctions. The maximal effect is obtained when TAP is added to both sides of the epithelium, and from variation in activity with pH the monoprotonated form is the active species. Cation permeability appears to be regulated in gall bladders by hydrated channels of some 10–20 Å in diameter, lined with ionised acid groups with pK_a values of around 4.4. TAP may be sufficiently small to enter these channels and block them by protonation of the acid groupings. The affinity of TAP is rather low ($4 \times 10^2\ M^{-1}$), and it is yet too early to assess whether this agent will be important in unravelling the properties of tight junctions.

CONCLUDING REMARKS

For the reader who has reached this far, it will be clear that the gulf which exists in our understanding of the process of ion translocation in lipid bilayers and in biological membranes is large. Progress in both areas will depend upon new technologies and new drugs. A major proportion of the drugs mentioned in this review are materials of natural origin. Perhaps there are more to be discovered, particularly in the sea, which will lead to seminal observations on ion translocation processes. Our inheritance from 'evolutionary chemistry' is being devoured at an irreplaceable rate, and diminishing returns can only be met by investment. In the concluding remarks to his excellent review, Narahashi [91] argues that we are capable of synthesising substances just as useful as those of natural origin. I believe that a fuller understanding of ion translocation processes will depend upon progress in medicinal chemistry.

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2 The Bio-Medical and Related Roles of Ion-Selective Membrane Electrodes

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INTRODUCTION

Glass electrodes have been standard items of laboratory equipment for about forty years, but the present widespread interest in ion-selective electrodes stems from the commercial availability in 1966 of the fluoride solid-state lanthanum fluoride electrode. Ion-selective electrodes are now commercially available for about twenty common anions and cations [1]. Their development has coincided with the demand for convenient methods of ion determinations in the biomedical field, stimulated by the successes of the fluoride electrode in determining trace fluoride in water fluoridation and dental health programmes.

Constructional principles of ion-selective electrodes are similar to the classical glass electrode, namely:

Internal reference element – Internal reference solution – Sensor membrane

However, certain solid-state membrane and coated-wire electrodes lack a conventional internal reference system and depend instead on direct wire contact to the back of the membrane.

Ion-selective electrodes may be classified according to membrane material into glass, homogeneous and heterogeneous solid-state, liquid ion-exchanger, carrier complex, semi-conducting organic charge–transfer complex and ion-radical salt, PVC matrix membrane with liquid ion-exchanger or other sensor, and electroactive hydrophobised graphite. Coated-wire, micro-, combination and enzyme electrodes are subsidiary to this classification as are the selective permeator membrane electrode systems for gases.

Detection limits of 10^{-5} mol dm^{-3} are attainable in most cases but some electrodes can better this by two or three orders of magnitude. However, even a 10^{-5} mol dm^{-3} solution is within the part per million (ppm) range, as for example 0.40 ppm for calcium, and 2.07 ppm for lead.

Interest in the use of ion-selective electrodes in the biomedical field is a natural consequence of the electrolyte composition of bulk body and cell fluids (*Table 2.1*), a proportion of which is in the ionised form. In extracellular fluids, sodium is the principal cation with chloride as the major anion. In intracellular fluids, potassium is the major cation and phosphate the principal anion – except in erythrocytes where chloride predominates. Of special interest is ionised calcium, because of its importance in various physiological and biochemical processes such as bone formation, nerve conduction, cerebral function, cardiac conduction and contraction, membrane phenomena, muscle contraction and relaxation, blood coagulation, and enzyme activation [2–4].

On the wider biomedical front, application of ion-selective electrodes takes in mineralised tissues, dental materials, enzyme reactions, pharmaceutical products and drug metabolism.

Table 2.1. INORGANIC ELECTROLYTE COMPOSITION (10^{-3} mol dm^{-3}) OF CERTAIN BODY FLUIDS [5,6]

Ion	*Plasma*	*Cell fluid (muscle)*	*Erythrocyte fluid*	*Sweat*
Na^+	153	10	15	10–80
K^+	5	148	150	7
Ca^{2+}	2.5	–	–	0.5–1.5
Mg^{2+}	1	20	1.5	0.0005
Cl^-	110	–	74	10–65
HCO_3^-	28	8	27	trace
HPO_4^{2-}	1.5	67	–	0.0005
SO_4^{2-}	0.5	–	–	<0.5

Investigations with glass electrodes for measuring pH are based on well-established principles and are not discussed here.

GENERAL ELECTRODE PRINCIPLES [1,6]

An ion-selective electrode is used as the sensing indicator electrode in a potentiometric cell assembly in the same way as a glass pH electrode where it contributes ($E_M + E_{M'}$) to the cell emf, E_{cell}, which comprises various junction potentials:

$$E_{cell} = E_M + E_{M'} + E_R + E_{R'} + E_j \quad (1)$$

E_M is the essential quantity in the measurement situation and corresponds to the potential arising at the junction of the ion-selective electrode membrane with the test or standardising solution. The remaining contributory potentials are assumed to remain constant so that E_{cell} is related to changes in E_M, but some of these can be troublesome, especially those of the reference electrode and related junction potentials.

When operating properly the ion-selective electrode potential, E, with respect to the reference electrode is given by

$$E = \text{constant} \pm S \log[a_A + k_{AB}^{pot}(a_B)^{z_A/z_B}] \quad (2)$$

According to Equation (2) the ion-selective electrode responds selectively to an ion, A, of charge z_A and activity a_A in the presence of an interfering ion, B, of

charge z_B and activity a_B; k_{AB}^{pot} is the selectivity coefficient. The constant term incorporates various junction potentials (except for E_M) as well as the standard potential characteristic of the ion-selective electrode. The second term on the right-hand side of Equation (2) assumes the positive sign for cations and the negative for anions. In most cases the calibration slope, S, approaches the Nernstian value of $2.303\ RT/z_A F$, and this holds over a wide p(ion) range.

Equation (2) allows for the fact that specificity is rather exceptional for ion-selective electrodes and further terms are required where the competition for electrode response is exerted by species additional to A and B. A common interference effect is the fall-off in linear calibration as illustrated by *Figure 2.1* (curve A). However, in addition to competitive response there can be other kinds of interference such as those that occur by the presence of complexing species reducing the true levels of the primary ions A.

Figure 2.1 also demonstrates a common method of obtaining the selectivity coefficient, k_{AB}^{pot}, whereby the emf response is measured for solutions containing a fixed amount of interferent with varying activities of the primary ion, A, for which the electrode is designed. This is known as the *mixed solution method with fixed amount of interferent* and k_{AB}^{pot} is calculated from

$$k_{AB}^{pot} = \frac{a_A}{(a_B)^{z_A/z_B}} \tag{3}$$

where a_A and a_B are values appertaining to the intercept of that part of the calibration curve (of near-zero slope) corresponding to complete interference by

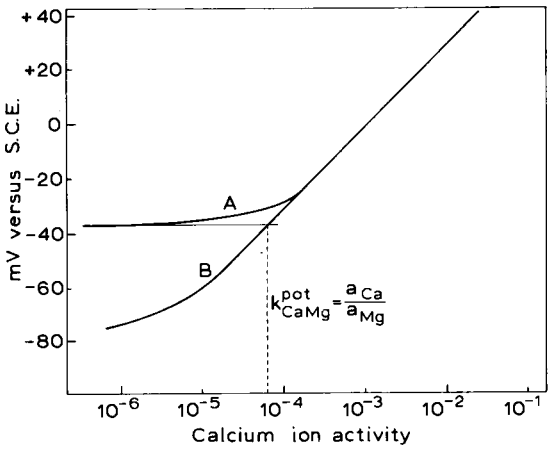


Figure 2.1. The effect of constant magnesium ion concentration on the response of the PVC electrode to varying calcium ion activities: A, 5 X 10^{-3} mol dm^{-3} Mg^{2+}; B, Ca^{2+} only

the interferent, B, with that of slope corresponding to more or less unfettered response by the primary ion, A. Generally, k_{AB}^{pot} values of less than unity calculated in this way imply that the electrode preferentially responds to the primary ion, A. However, the power term $(a_B)^{z_A/z_B}$ in Equation (3) calls for caution in exercising such a generality [7].

The selectivity coefficient, k_{AB}^{pot}, frequently varies with the level of interferent and k_{AB}^{pot} values are more meaningful if the activity of the interfering ion for which they were determined is also quoted. It is then a simple matter to calculate by Equation (3) the useful limit of the electrode for ion A.

An alternative mixed-solution method for expressing selectivity involves varying the interferent activity, a_B, at constant primary ion activity, a_A. This method is normally used for expressing the pH range over which ion-selective electrodes are useful.

INTERFERENCE ELIMINATION

The application of ion-selective electrodes, especially with biomedical systems, can become complicated owing to the presence of colloidal electrolytes, the loss of carbon dioxide, and the complexation equilibria by buffers and other adjusters. Such complications hinder routine use in the clinical laboratory and measurement by the electrodes of the activity rather than the concentration of ions also acts as a psychological barrier. Clearly, these devices demand a higher level of technical competence than, say, the glass electrode for pH measurements, but this can be compensated by access to data on ionic levels that are becoming increasingly recognised as the real variables of interest in biochemical and physiological situations.

Interference can frequently be overcome; for example, low pH buffer systems containing silver sulphate and aluminium sulphate in nitrate determinations [8], maintain the equilibrium bicarbonate low, keep the water-extractable organic acids undissociated, remove chloride as silver chloride and complex anions of organic acids with aluminium.

Electrode response is affected by ionic strength variations because of the associated effect on activity coefficients [9]. This makes an assessment of ionic strength on the test solution desirable. However, because of the powered term in $\mu = \frac{1}{2}\Sigma Cz^2$, conductance monitoring may not always be satisfactory, unless, of course, the individual ionic contributions (concentrations, C, and valence, z) are known from previous analyses. Such information is useful for preparing calibration standards of the same ionic strength range as the sample under examination and is the basis of synthetic sea water samples as calibrants. Calcium chloride standards in 0.150 mol dm^{-3} sodium chloride is a similar ploy used in calibrating

calcium ion-selective electrodes for serum-plasma calcium ion determinations in the 0.5×10^{-3}–2×10^{-3} mol dm^{-3} range.

Situations where the ionic strength can be mimicked are frequently not the rule for there can be considerable overall variation in composition between samples. In these cases, it is helpful to add an 'ionic strength adjuster' to both sample and calibrating solutions in order to achieve a common ionic strength level, to release the ion of interest from complexes, and to buffer samples within regions of minimal pH interference.

Although virtually any ionic substance may function as an ionic strength adjuster, it must not complex with the primary ion, and the selectivity coefficient, k_{AB}^{Pot}, must be negligible – B in this case being the ionic strength adjuster constituent. Several systems have been devised and a well known one is TISAB (Total Ionic Strength Adjustment Buffer) used in fluoride determinations [10]. With a pH value of about 5, it is composed of 1 mol dm^{-3} sodium chloride, 0.25 mol dm^{-3} acetic acid, 0.75 mol dm^{-3} sodium acetate and 1×10^{-3} mol dm^{-3} sodium citrate. Defects of ionic strength adjusters have been reported and attributed in TISAB to the carboxylate content [11]. As a result, citrate in TISAB has been replaced [12] by DCTA (1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetra-acetic acid) although it has been shown [13] that the lanthanum fluoride electrode is only minimally affected by acetate and citrate.

MEASUREMENT METHODS

The above implies *simple measurement of the ion-selective electrode potential, E*, with respect to a suitable reference electrode and relating the potential to activity or concentration by a calibration graph as the means of using ion-selective electrodes. Use in *potentiometric titrations* depends on the change in *E* with volume of added titrant. There are, however, other techniques by which ion-selective electrodes may be used; these include differential and null-point potentiometry.

Differential potentiometry is a concentration cell technique involving the use of a matched pair of electrodes whose liquid junction potentials become negligible when a sufficiently large excess of an inert electrolyte is used [14]. The unknown solution is placed in one half-cell and a standard solution in the other. The potential difference is related to the ion concentration by a calibration curve.

The precision of the differential potentiometric method can be improved by using *null-point potentiometry* [15–19]. The ion concentration is measured, not from a single potential reading, but by adjusting the composition of one of the half-cell solutions to match the other until a potential (the null, or bias poten-

tial) is obtained that corresponds to that prevailing when both half-cells contain an identical solution.

Known addition, known subtraction and Gran's plot methods are other ways of using ion-selective electrodes [14,20–27]. In the *standard addition to sample method,* the potential, E_0, of the ion-selective electrode with respect to a suitable reference electrode is measured for the sample solution of volume, V_0, and total concentration, C_0, of the sought species:

$$E_0 = \text{constant} \pm S \log x_0 f_0 C_0 \tag{4}$$

where S is the calibration slope, f_0 is the activity coefficient, and x_0 the fraction of uncomplexed ions. A new potential E_a is measured after addition of a small volume, V_a, of a standard solution (concentration C_s) of ions of the species sought, where $C_s \approx 100C_0$

$$E_a = \text{constant} \pm S \log x_a f_a \frac{(V_0C_0 + V_aC_s)}{(V_0 + V_a)} \tag{5}$$

where f_a and x_a correspond to the new activity coefficient and fraction of free ions, respectively. An essential assumption is that $x_0 \approx x_a$ and $f_0 \approx f_a$. Hence, the difference between the above two equations simplifies to

$$E_a - E_0 = \Delta E = \pm S \log \frac{(V_0C_0 + V_aC_s)}{C_0(V_0 + V_a)} \tag{6}$$

and C_0 can be resolved

$$C_0 = \frac{C_s}{10^{\pm \Delta E/S}(1 + V_0/V_a) - V_0/V_a} \tag{7}$$

or for no allowance for volume change, that is, V_a small in relation to V_0

$$C_0 = \frac{C_sV_a}{(10^{\pm \Delta E/S} - 1)V_0} \tag{8}$$

For *analate addition* purposes [23], that is, sample addition to standard, the corresponding version of Equation (7) is

$$C_0 = C_s[10^{\pm \Delta E/S} \cdot (1 + V_s/V_{0a}) - V_s/V_{0a}] \tag{9}$$

where V_{0a} is the volume of sample solution added.

The *known subtraction method* depends on lowering the level of uncomplexed ions by adding a complexing agent when the structures of the equations depend on the nature of complexation (or precipitation), that is, 1 : 1 or 1 : n.

An interesting variation of the standard addition method depends on a modification of the method described by Gran [25] for presenting potentiometric titration data in linear form using a semi-antilog plot. The principle is apparent in Equation (5) above for which the antilog version is

$$(V_0 + V_a)\, 10^{\pm E_a/S} = x_a f_a\, 10^{\pm \text{constant}/S}(V_0 C_0 + V_a C_s) \tag{10}$$

where V_a is the volume of standard solution added. A plot of the left-hand side of Equation (10) as ordinate versus V_a gives a straight line to intercept the abscissa for a V_0 value where

$$C_0 V_0 = -C_s V_e \tag{11}$$

and from which the concentration of the ion under test, C_0, can be easily calculated.

Computation of the left-hand side of Equation (11) is avoided by using semi-antilog Gran's Plot Paper supplied by Orion Research Incorporated which has been corrected for limited volume changes, that is, for $(V_0 + V_a)$, and which assumes sufficiently dilute solutions and that $x_a = 1$. Hence, all that is required is to plot the emf response on the ordinate against the volume of standard addition on the abscissa when the intercept, V_e, is easily discernible.

It is an easy matter to design a ruler to take in the left-hand side of Equation (10) for the direct plotting on ordinary graph paper of ordinate data from E_a readings. This ruler can be appropriately biased for planned volume changes. However, both the Orion Gran's Plot Paper and rulers are geared to fixed response patterns. This is Nernstian for Orion Gran's Plot Paper, but rulers can be made for a variety of electrode calibration slopes.

ACTIVITIES AND ACTIVITY COEFFICIENTS

Apart from true interference, all ions to various degrees affect the ionic strength of solutions and hence the activity of the primary ions, A, to which the appropriate ion-selective electrode respond. The difficulties of developing ion-activity scales for ion-selective electrode measurements to match the success of the pH scale have formed a barrier to the adoption for routine measurements of these devices and particularly for the complex mixtures found in biological fluids. The problem has been recognised [28] and attention has been given to calibrating

Table 2.2. COMPARISON OF pM AND pX VALUES OF DIFFERENT CONVENTIONS [28]

Salt	*Debye–Hückel convention*		*MacInnes convention*		*pH convention*	
	pM	*pX*	*pM*	*pX*	*pM*	*pX*
KCl	1.114	1.114	1.114	1.114	1.118	1.110
NaF	1.116	1.116	1.106	1.126	1.108	1.124
NaCl	1.110	1.110	1.106	1.114	1.108	1.110
$NaClO_4$	1.111	1.111	1.106	1.116	1.108	1.114
$CaCl_2$	1.898	1.282	1.880	1.291	1.887	1.286

Data refer to $\mu = 0.1$ and $t = 25°C$.

ion-selective electrodes on a concentration basis since this is the medically understood quantity [9,29].

On the calibration of ion-selective electrodes, an early and simple recommendation [30] was based on their general (but not always, because of complexation and other phenomena) independence of the nature of the co-ion and standardisation in solutions of the completely dissociated chloride salts for cation response and in solutions of the completely dissociated sodium salts for anion response. Among the exceptions was the recommended calibration of fluoride ion-selective electrodes with potassium fluoride rather than sodium fluoride, which is likely to become associated in moderately concentrated solutions [31].

The practical matter of choosing an arbitrary 'conventional' means of evaluating the activity of an individual ionic species that cannot be exactly defined may be discussed from the standpoint of dilute solutions, concentrated solutions and mixtures of electrolytes [1,28]. In *dilute solutions*, multiple pathways to the activity coefficients, f, of single unassociated ions exist and as can be seen from *Table 2.2*, the values of pM and pX obtained by various simple conventions do not differ greatly at an ionic strength of 0.1.

In *concentrated solutions*, the matter of choosing the most suitable pathway increases in importance and large errors are possible in practical measurements as a result of residual liquid junction potentials. Further, when the cation is hydrated in solution, the activity coefficient frequently passes through a minimum in the molality range 0.5–1.0 mol kg^{-1} and thereafter rapidly becomes greater than unity [28,32]. To counter the greater problems at higher ionic strengths, Bates, Staples and Robinson [33] used the Robinson–Stokes hydration theory as the basis of a convention for single ionic activities. This means that individual variations in ionic activity at a given ionic strength are accounted for

in terms of individual ionic hydration numbers, h, assigned on the assumption that the chloride ion has zero hydration number. Thus, by applying the thermodynamics of electrolyte solutions, equations have been developed [31,33] for splitting mean activity coefficients into the contributions of the individual ions and which satisfy the conditions [28] of (i) being consistent with the MacInnes, Debye–Hückel, and pH conventions at ionic strengths of less than 0.1; (ii) allowing for specific differences in the properties of ions in concentrated solutions; and (iii) recognising that the activity coefficient of a given ion is not dependent solely upon the ionic strength but varies with the composition of the solution. For a univalent electrolyte [28,30,33] MX:

$$\log f_{M^+} = \log f_{\pm} + 0.00782\,(h_M - h_X)\,m\phi \tag{12}$$

and

$$\log f_{X^-} = \log f_{\pm} + 0.00782\,(h_X - h_M)\,m\phi \tag{13}$$

where ϕ is the water activity (osmotic coefficient) and m the molality.

The convention has succeeded in yielding ionic activities that agree with 'observed' values [34] and has also been extended with equal success to special cases of mixtures of two univalent electrolytes [35,36]. Of course, in practice, one of the attractive features of ion-selective electrodes is their ability to respond to the activity of one particular ion in the presence of others in *mixed electrolyte systems*, so that a knowledge of ion activities in mixtures is of some importance. Here, most interest has centred on mixed electrolyte solutions of biological significance [2,9,28,30,34–44], but whatever the system, the primary concern is the variation in activity of one electrolyte in the presence of others.

The interaction between ions, mainly between those of opposite charge, can lead to a variation of activity coefficients with the composition of mixed electrolyte solutions and thus contradict the Debye-Hückel formula prediction that log $f_{\pm}$ is proportional to $\mu^{1/2}$. In dilute solutions the conflict is minimal, but at higher concentrations ($>$0.1 mol dm^{-3}) the influence of ion interactions can, for example, alter the significance of the ionic size paramerer, d, in the first term of Equations (12) and (13), namely:

$$-\log f_{\pm} = \frac{Az_+z_-\mu^{1/2}}{1 + Bd\mu^{1/2}} \tag{14}$$

Studies with an Orion 92-20 calcium ion-selective electrode on mixed calcium chloride–calcium nitrate solutions and with nitrates and chlorides of sodium and potassium have shown that within experimental error, the mean activity coefficients of calcium chloride in mixed solutions up to an ionic strength of 0.3 mol

dm^{-3} are equal to those which would hold for pure calcium chloride of the same ionic strength [44].

Mean sodium chloride activity coefficients have been determined [41] with a sodium ion-selective electrode and silver–silver chloride reference electrode system in mixed sodium chloride–calcium chloride solutions within the range of sodium chloride and calcium chloride levels (0.05–0.5 mol dm^{-3}) encountered in extracellular fluids. These show that at constant ionic strength, $\log f_{NaCl}$ varies linearly with the ionic strength of calcium chloride in the mixture in accordance with Harned's rule [45]:

$$\log f_{NaCl(mixture)} = \log f_{NaCl(pure)} - \alpha_{12}\mu_{CaCl_2} \tag{15}$$

where $-\alpha_{12}$ is a constant represented by the slope in a plot of $\log f_{NaCl(mixture)}$ against μ_{CaCl_2}. The activity coefficients of sodium chloride in solutions containing 5×10^{-3} mol dm^{-3} calcium chloride (0.5 mol dm^{-3} total ionic strenth) were shown to be close to those of corresponding pure sodium chloride solutions, that is, the effect of calcium chloride on sodium chloride activity in extracellular fluids would be negligible [41]. The reverse is not the case, for calculations involving the alternative version of Harned's rule:

$$\log f_{CaCl_2(mixture)} = \log f_{CaCl_2(pure)} - \alpha_{21}\mu_{NaCl} \tag{16}$$

show that the activity coefficients of calcium chloride in mixtures typical of extracellular fluids were considerably less than those of corresponding pure calcium chloride solutions [41].

Comparatively low levels of other ionic components in serum, like potassium and magnesium which comprise less than 6 per cent of the total ionic strength of blood serum, have a negligible effect on the activity coefficient of calcium ions at ionic strengths below 1.0 mol dm^{-3} [38]. The activity coefficients of these ions can be expected to be affected by the high sodium level as with calcium.

In conclusion, the evaluation of individual ionic activities in multicomponent electrolyte mixtures poses difficulties, but the ionic strength of body fluids is sufficiently low ($\approx$0.16 mol dm^{-3}) so that the actual single ion activity coefficients are not expected to be too far removed from the values derived from conventions that have proved useful at ionic strengths of 0.1 mol dm^{-3} and less [28,30]. On this basis, Mohan and Bates [9] have examined the behaviour of pH, sodium glass, valinomycin and the Simon calcium electrodes in a set of calibrating standards with the main inorganic constituent compositions paralleling the range of concentrations normally encountered in blood serum in clinical analysis. They conclude that these electrodes can be calibrated on a concentration basis and suggest that the mixtures they used may serve as calibrating standards in clinical analysis.

BODY AND TISSUE FLUIDS

Body fluids are complicated mixtures whose inorganic electrolyte composition in plasma and muscle cells is normally fairly constant, but which may show considerable variations in gastric and pancreatic juices, sweat, saliva and urine. Ion-selective electrodes are the only devices capable of measuring the important normal ions and dissolved gases in fluids and they have proved particularly useful for medical, biochemical and physiological investigations of calcium, potassium, sodium, ammonium, chloride and fluoride. To a lesser extent, the electrodes have been used for bromide, iodide, lead, carbon dioxide, enzymes, proteins and in metal–nucleotide systems.

SERUM, PLASMA AND WHOLE BLOOD CALCIUM ION STUDIES

Calcium levels in plasma and serum are closely controlled by several homeostatic mechanisms so that only accurate and precise determinations of calcium will reveal a loss of steady-state control and yield useful diagnostic information [3]. Because of this and as already mentioned above, determination of the physiologically active ionised calcium is highly desirable.

The ionic or 'free' calcium in plasma and serum is associated mainly with chloride ions [46] and represents the calcium that is not bound to protein (normally 30–55 per cent and non-diffusible) or other complexing species such as phosphate, citrate or bicarbonate (normally 5–15 per cent and diffusible). In the average healthy person, the calcium ion level is just below one-half of the total and is so closely regulated that no gross variability can be detected [47]. Furthermore, even in the absence of an adequate calcium intake and a negative calcium balance, plasma calcium remains unchanged for a considerable length of time as the calcium stores in bone are called upon [3,48].

The physiological role of the ionised form of calcium was brought into prominence by the ingenious bioassay of McLean and Hastings [49–51] which was based on the contractile response of isolated frog hearts to varying levels of calcium ion perfusates. This has emphasised that the measurement of ionised calcium is a very useful diagnostic aid as in primary hyperparathyroidism where, in most cases, the calcium ion level is raised even when the total calcium may be normal [52]. However, bioassay methods are difficult to perform while later chemical methods based on the colour reaction of calcium ions with murexide dye is too pH-dependent with the added complication of complexation between murexide and albumen and heavy metals, requiring the separation of proteins and heavy metals before analysis [53]. It is not surprising, therefore, that much interest centres on the calcium ion-selective electrode. In all its applications, it is second only to the fluoride electrode, and its usefulness in serum or plasma

calcium ion studies is supported by the agreement between 'normal' ionic calcium obtained with the electrode (1.00–1.26 mmol dm^{-3}) and the McLean and Hastings [51] frog heart method (1.06–1.31 mmol dm^{-3}).

Calcium ion measurement

When using ion-selective electrodes, and indeed any measuring device, proper technique and an appreciation of principles, scope and limitations are prerequisites to the best results, otherwise there is the dismay expressed by some investigators. With ion-selective electrodes as with pH electrodes, the property measured (the potential) is proportional to the logarithm of the level of active species so that high precision is rarely achieved in direct measurements. In addition, the problems of liquid junction phenomena, laboratory conditions and lack of stability with many electrodes may make readings to within ±0.1 mV difficult. With bivalent ions such as calcium, even readings to within ±0.1 mV lead to uncertainties of ±0.8 per cent (0.4 per cent for univalent ions) under Nernstian conditions. If the error is random it can, in principle, be reduced with replicate determinations [1].

With drifts of 1 mV, the error for bivalent ions will be 8 per cent. This emphasises that attention must be given to electrode quality, frequent standardisation and use of high precision emf-measuring equipment, especially since the full range of 'normal' serum calcium ion levels at approx. 0.9–1.5 mol dm^{-3} extend over an emf range of just 6.6 mV.

There have been complaints over expensive and cumbersome membrane replacement with liquid ion-exchanger electrodes, but such technical inconveniences can be minimised by confining the liquid ion-exchanger or carrier sensors in PVC matrix membranes [6,24,54]. Such practical considerations with the above attention to principles and proposals [9] for concentration calibration of ion-selective electrodes for use in biological fluids can all facilitate the biological scientist's confident use of ion-selective electrodes.

Much of the uncertainty concerning activity coefficients and liquid junctions can be minimised by calibrating the ion-selective electrode with calcium chloride standards (approx. 0.5–0.2 mmol dm^{-3}) in 0.150 mol dm^{-3} sodium chloride solutions. This corresponds to an emf span of just approx. 18 mV, thus emphasising the need for using precise emf-measuring instruments and attention to electrode care and solution details.

Liquid junction problems can be avoided by using as reference a solid-state lanthanum fluoride membrane electrode in conjunction with spiked fluoride samples [9]. On details, Moore [46,53] has highlighted the need for careful sampling and subsequent processing by his studies on technique, selectivity, calibration, sample freezing and pH.

Providing the pH value is above 5.5, there is no interference from hydrogen ions. In this respect, the pH of venous blood is of the order of 7.3–7.4, but the buffering effect of carbon dioxide can cause difficulties – a loss apparently causing a decrease and a gain an increase in ionic calcium, respectively [53]. This also calls for caution in the use of buffers unless their effects on calcium ionic activity is known. Some authors have added trypsin and triethanolamine buffer to their ionised calcium standards [3].

The pH effects on heparinised whole blood resemble those in corresponding sera, with a fall in pH leading to an increase in ionic calcium. However, the ionic calcium level is less in whole blood than in serum [53], and even at the normal venous pH of 7.3–7.4, the difference is significant, being 0.045 mmol dm^{-3} at pH 7.32. This depression is accountable by a calcium–heparin complex. Although small amounts of heparin (up to 30 units per cm^3 of blood) are claimed to have no significant effect on calcium ion activity, indiscriminate use of heparin should be avoided when collecting whole blood samples.

Temperature-induced variations in protein binding affecting ionic calcium levels support the use of thermostatted flow-through calcium ion-selective electrodes and there is an abundance of electrode and electrolyte data at room temperature to favour determining ionic calcium at 25°C rather than at 37°C. In this respect, ionic calcium can be reliably determined in previously frozen serum [53,55] and sera stored frozen in 1 cm^3 plastic insulin-type syringes for one to three days showed no significant changes in pH or ionised calcium concentration [55]. This is helpful for sera are often not analysed immediately after collection. An alternative storage with sera kept frozen under oil with or without re-equilibration with carbon dioxide is associated with significant changes in both pH and ionic calcium [55].

Calcium ion investigations

The majority of calcium ion investigations concern 'normal' and pathological sera and plasma with discussion leading to the relation between the total and ionic forms [3,46,47,52,53,55–135]. The data in *Table 2.3* illustrate the general agreement variously reported for human sera, plasma and blood samples although the values are somewhat dependent on the type of electrode used (usually Orion flow-through or static models but PVC matrix membrane electrodes have their advantages [54]) as well as pH, temperature, age, sex and sampling time of donor's sample. There can be differences between populations and within populations according to state of health, but for any given individual, Moore [53] found a variation of only 6 per cent in the serum ionic calcium level over several months. However, even that could account for the reported lack of correlation of ionic to total calcium in 23 normal adults [59].

Table 2.3. TOTAL AND IONIC CALCIUM IN HUMAN SERUM, PLASMA AND BLOOD *

Sample	*Total ** calcium (mmol dm^{-3})*		*Ionised calcium (mmol dm^{-3})*		*Ionised/ Total (× 100)*	*No. of subjects for ionised calcium*	*Ref.*
	Mean	*Range (± 2 S.D.)*	*Mean*	*Range (± 2 S.D.)*			
Serum	2.41	0.56	1.18	0.31	49.2	17	74
	2.45	0.24	0.99	0.08	40.5	23	59
	2.43	0.14	1.26	0.06	52.2	11 (F)	77
	2.45	0.14	1.21	0.09	49.4	10 (M)	
	2.43	0.04	1.18	0.03	48.6	15	103
	–	–	1.22	0.05	–	147(M)	86
	–	–	1.24	0.06	–	150(F)	
	2.50	0.16	1.08	0.06	43.2	84	102
	2.41	0.10	1.10	0.06	45.6	22	76
	2.48	0.29	1.14	0.13	45.8	70	53
	3.04	–	1.39	–	45.7	2	135
	2.64	0.42	1.21	0.25	45.6	16	78
	2.29	0.24	1.22	0.09	53.4	231 for total 397 for ionised	79
	2.44	0.08	1.25	0.03	51.24	20	81
Plasma	2.43	0.11	1.00	0.06	41.2	22	76
	2.65	0.41	1.16	0.22	43.8	50	80
	2.71	0.06	1.31	0.02	48.2	8	78
	–	–	0.94	0.03	–	20	136
Whole blood	–	–	1.08 (estim.)	–	–	6	53
	2.72	0.03	1.34	0.02	49.2	8	80
	1.42	0.05	1.11	0.02	46.7	13(A)	
	2.45	0.04	1.09	0.02	44.4	7(C)	
	2.60	0.04	1.24	0.02	47.7	23(N)	
	–	–	0.94	0.03	–	20	136

* Ionic levels determined with calcium ion-selective electrode.
** Determined by spectroscopic, EDTA titration or colorimetric techniques.
F, females; M, males; A, adults; C, children; N, newborns.

Except for the data of Seamonds, Towfighi and Arvan [102] each taken at 37°C, the data of *Table 2.3* refer to temperatures between ambient and 27°C. This is interesting because of the tendency for the hydrogen-ion activity to increase at higher temperatures with an expected decrease in the fraction of calcium that is bound [3]. Moore [53] found little variation with temperature with the mean ionic calcium of two groups of normals being 1.161 and 1.169

mmol dm^{-3}, respectively, at 25 and 37°C. By contrast, Hansen and Theodorsen [90] found values at 37°C to be, on average, 0.02 mmol dm^{-3} lower than at 24°C while the serum ionic calcium data of Seamonds, Towfighi and Arvan [102] in *Table 2.3* are, except in one instance, lower than the remaining mean ionised calcium data quoted.

Trypsin and triethanolamine buffer in ionised calcium standards are said to decrease equilibration time and to lead to better electrode reproducibility [3,59,86] and the serum data corresponding to references 59, 79 and 102 in *Table 2.3* refer to electrodes calibrated with such buffers added to the usual calcium chloride standards containing 0.150 mol dm^{-3} sodium chloride. However, such buffered standards must be prepared immediately before use with highly purified trypsin and filtered immediately so as to remove undissolved trypsin [55,59,80].

The investigations on calcium levels in sera are interesting in their scope. For example, total and ionic calcium levels have been measured in 147 normal males and 150 normal females aged 17–93 years [86]. The ionic calcium in females was slightly higher (1.24 mmol dm^{-3}) compared with the male samples (1.22 mmol dm^{-3}) as was similarly observed by Robertson and Peacock [77] with a much smaller sample (*Table 2.3*). Ionic serum calcium did not vary with time of day, time since last meal, or body position [86]. The decrease in total and ionic calcium with age was statistically significant for males but smaller and borderline in females [86]. However, it is hazardous to draw firm conclusions from these trends especially since other investigators [71] found no significant variation in ionic calcium by sex or age group (20–30 and 55–65 years).

The total and ionic maternal serum calcium levels of 54 women with normal uncomplicated pregnancies were each significantly less than in normal cord serum collected from several umbilical cords and prior to placental separation. This is accounted for in terms of simple diffusion rather than increased protein binding [121]. The calcium ion-selective electrode has also established the existence of hypocalcaemia during normal uncomplicated pregnancies [122], the ionic serum calcium being 1.11 ± 0.03, 1.10 ± 0.02 and 1.05 ± 0.01 mmol dm^{-3}, respectively, during the first, second, and third trimesters. Hypocalcaemia has also been studied in relation to recurrent apnea in premature infants [124].

Hypocalcaemia is associated with hypo- and pseudohypoparathyroidism while hypercalcaemia commonly relates to cirrhosis, adrenal insufficiency, osteoporosis, sarcoidosis, disease atrophy, and hyperparathyroidism. Calcium assays are rather important diagnostic aids especially since a routine screening on total serum calcium of 50 330 patients revealed 55 cases of unsuspected primary hyperparathyroidism [137] and the disease is on the increase in the U.S.A. and Sweden [138]. In primary hyperparathyroidism, the glands become autonomous and secretions of parathyroid hormone (PTH) are no longer controlled by the

plasma calcium. It is claimed that immunoassay for PTH and ionic calcium level are more important diagnostic tests for the disease than total serum calcium [139], although total calcium and inorganic phosphorus assays are also useful [46]. In general, endocrinologists and endocrine surgeons recommend [138] exploration of patients with sera of total calcium reaching 3–3.2 mmol dm^{-3}. Hines and Suker [140] have studied 51 patients with proven hyperparathyroidism, several of whom exhibited low serum inorganic phosphorus and elevated serum calcium. As an example of this trend, the total calcium level of 4.45 mmol dm^{-3} in the serum of a 48-year-old Caucasian female fell to 2.25 mmol dm^{-3} following surgical removal of a parathyroid adenoma and later stabilised at 2.37 mmol dm^{-3}.

There are occasions when the total serum calcium can be normal as in 'normocalcaemic' hyperparathyroidism when the raised ionic calcium can aid diagnosis as well as provide a differential diagnosis from idiopathic hypercalciuria which is the greatest single cause of renal stone formation and is characterised by high urinary calcium but normal serum calcium [52,57]. Thus, five patients with sera of normal total calcium showed elevated ionic calcium (1.42–1.50 mmol dm^{-3}) and the plasma showed a marginal elevation of PTH concentration. Also, neck exploration revealed parathyroid adenoma and four of the patients had high urine calcium [57]. In each case, parathyroidectomy was followed by reduction in ionic and total serum calcium levels and in urinary calcium [57].

On the wider scale, ionised calcium in sera measured with the calcium ion-selective electrode have helped in studying the direct effect of calcium on the hyperparathyroidism of chronic renal failure when it was shown that rats on low calcium diet developed larger parathyroids and more severe bone disease at the end of four weeks while diets with above normal calcium levels produced no additional benefits [130]. Other studies include the relation between hypercalcemia and normal ionised serum calcium in a case of myelomatosis [86], the detection of hypocalcemia in susceptible neonates [68], and studies on serum ionised calcium changes following citrated blood transfusion in anaesthetised subjects [93]. The transfusion studies showed six patients during anaesthesia to have a decrease of 0.135 mmol dm^{-3} after 500 cm^3 and 0.15 mmol dm^{-3} after 1000 cm^3 blood, respectively. However, the calcium ion concentration increased by a mean of 0.075 mmol dm^{-3} in 10 min following complete infusion of the blood [93].

The influence of hormones, drugs and miscellaneous compounds on serum and plasma ionised calcium has also been investigated [70,107,119,120,125]. The Orion 99-20 flow-through model calcium ion-selective electrode is especially suitable for this purpose in anaesthetised patients and animals. Twice-daily doses (50 mg) of hydrochlorothiazide for 25 days administered to 9 normal human subjects significantly raised total and ionic calcium levels. The effect lasted for a

few weeks after discontinuing the diuretic drug without any significant correlation between ionic calcium and total protein [125]. Streptomycin and related antibiotics do not change ionic calcium in pooled human sera at any pH from 6.0 to 8.0. Oral doses of phosphate lowered total and ionised calcium but raised parathyroid hormone by 60 to 125 per cent within one hour [83].

Citrate intoxication, known to follow rapid transfusion of stored blood, is a common source of hypocalcemia in critically ill man and it has been recommended that calcium replacement therapy be undertaken only with close monitoring of ionic calcium with ion-selective electrodes [141]. The calcium ion-selective electrode has also been used [142] to monitor calcium ion levels in recipient's blood when it was observed that addition of calcium chloride to the circulation *following* administration of acid–citrate–dextrose blood caused a sharp rise and then a fall in the recipient's level of ionised calcium.

Monkeys are considered the best substitutes for humans in meaningful studies of calcium metabolism wherein ionised calcium is hormonally controlled within normal limits by parathyroid and thyrocalcitonin. Low doses of thyrocalcitonin administered to *Macaca cynomologus* only lowered total calcium whereas higher doses depressed both total and ionic calcium levels [120]. Thyrocalcitonin also lowered total and ionic calcium in the serum of intact rats but not to the same extent as after parathyroidectomy. Parathyroid hormone extracts given to the same parathyroidectomised group elevated both total and ionised calcium [70]. Another study on parathyroidectomised rats revealed a depression of all three serum calcium fractions (protein-bound, diffusible-complexed and ionic) which was reversed on administration of parathyroid hormone.

Clearly, the assay of ionised calcium in serum by ion-selective electrode is establishing itself clinically and the Orion Model 99-20 flow-through electrode and more recently the model SS-20 are prominent in this. The extent of the applications can be strengthened by the simple, straightforward construction and maintenance principles of the PVC matrix membrane electrode based on calcium dialkylphosphate or other suitable sensor and mediator [24].

OTHER ION (AND AMMONIA) STUDIES IN SERUM, PLASMA AND BLOOD

Sodium, potassium, ammonium and ammonia

The possibility of alkali metal ion-binding can be dismissed on the basis of sodium and potassium ion studies [26,36,139–145]. Glass electrode studies on serum indicate a correlation with flame photometer sodium levels amounting to no sodium binding [2,143]. One premise for this is the assumption of serum water content being 96 per cent by volume giving a mean f_{Na^+} of 0.747 com-

pared with 0.750 for f_{NaCl} in pure solution at a sodium ion concentration of 0.140 mol dm^{-3} [26].

Earlier glass electrode studies for potassium have given conflicting results [2], but serum is an unfavourable fluid for accurate potassium glass electrode measurements since the sodium : potassium ratio is of the order 35 : 1 to 40 : 1. To counter this, the selectivity for potassium ions over sodium ions is only about 10. Such interference problems have been overcome by the availability of liquid ion-exchanger and valinomycin electrodes that are highly selective to potassium in the presence of sodium, and their function over a large pH range makes buffering unnecessary. Independent studies with these electrodes agree with potassium levels ($\approx$4.5 mmol dm^{-3}) determined by flame photometry and atomic spectroscopy and confirm minimal ion-binding [143–147].

On the matter of correlations between electrode and spectroscopic data, there can be some bias but while electrode potassium values are very slightly higher in one study [145], it is the flame photometer potassium data that are higher in another [148]. The bias in the second example [148] was attributed to faulty electrode calibration.

Nitrogen occurs in various forms in biological fluids and certain electrodes are suitable for at least three of these, namely, ammonium, ammonia, proteins (including enzymes) and urea. By way of illustration of possible applications, direct measurement of ammonia in serum and blood is clinically interesting since high levels are encountered in hepatic coma and may also indicate the nature of gastro-intestinal haemorrhage in accident cases. The ammonia gas electrode [149] and the air-gap electrode [150] are suitable for this purpose.

The ammonia gas electrode gave 2.0 ($\pm$0.5) $\times 10^{-5}$ mol dm^{-3} ammonia nitrogen in the whole blood of 35 normal patients after protein precipitation compared with 1.9 ($\pm$0.5) $\times 10^{-5}$ mol dm^{-3} obtained by an established ion-exchange resin method and with a correlation coefficient of 0.994 [151]. Good agreement between the ammonia electrode and the ion-exchange method was also obtained in 9 cases of liver disease where the ammonia nitrogen levels ranged from 3.2 $\times$ 10^{-5} mol dm^{-3} to 19.7 $\times 10^{-5}$ mol dm^{-3}. Semicomatose conditions appeared in 2 patients at about 10.7 $\times 10^{-5}$ mol dm^{-3} and one case became comatose at 13.7 $\times 10^{-5}$ mol dm^{-3} ammonia nitrogen [151].

On interferences of the ammonia electrode, the effect of amines like methylamine [151] and trimethylamine [152] is quite noticeable and a simple correction in the latter case was made by adding a 1 : 1 formalin sample to eliminate ammonia and obtain the trimethylamine reading [152]. Amphetamines and antihistamines have no effect on the electrode [151].

Alexander and Rechnitz [153–155] have used a silver sulphide electrode for the direct determination of changes in total protein level in blood serum as well as albumin : globulin ratios, kinetics of protein denaturation and analysis of

serum filtrates [153] and for automated protein analysis [154]. As little as 0.5 μg cm^{-3} of antigen can be quantitatively determined without interference from other serum proteins. It has been suggested that this technique could be applied to the wide range of immunoassays now possible with available antisera and in turn simplify classical colorimetric procedures [155].

Chloride and fluoride

Although chloride is the principal anion in blood fluids, there are just a few instances of chloride ion-selective electrodes having been used for such assays [156–158] but the most important role of this electrode is for screening sweat chloride levels in cystic fibrosis diagnosis [159].

Fluoride, on the other hand, is a trace element in animals and is normally present in blood serum at about the 5×10^{-7} mol dm^{-3} level, but this can be considerably enhanced in patients anaesthetised with certain fluorocarbons and in the employees of certain important industries. The recent interest in fluoridation of water supplies has also focused attention on serum fluoride, but a difficulty here is access to low fluoride serum for preparing standards. Hence, human serum from a normal young adult who has not drunk fluoridated water for 24 h is acceptable when the inorganic fluoride will be $\leqslant 5 \times 10^{-7}$ mol dm^{-3} [160]; fluoride-free water may also be used with little loss of accuracy.

The fluoride ion-selective electrode may be used directly for free inorganic fluoride, while total fluoride is usually obtained after ashing or Schöniger flask combustion, the higher values for the latter indicating that a proportion of the fluorine is bound [160–167].

Taves [164] has used renal clearance methods using fluorine-18 to obtain serum with a predicted 1.05×10^{-7} mol dm^{-3} fluoride and has shown the fluoride ion-selective electrode results to agree with those obtained by the fluorescence of a Morin–thorium complex. The electrode readings were taken on neutralised diffusates, a common method of isolating and concentrating fluoride but over which an alternative reverse extraction approach has recently been proposed [168].

A large portion of the fluoride in rat, steer and human sera is ultrafilterable [169] and bound but not to protein of molecular weights exceeding approx. 25 000. The mean ionic fluoride of $1.0\ (\pm 0.3) \times 10^{-6}$ mol dm^{-3} in 10 aliquots of bovine ultrafiltrates was independent of pH between 4.51 and 5.50 and moreover, it did not increase on adding citrate ligand thus indicating the absence of metal–fluoride complexation. Ultrafiltrates of 18 adults from a low waterborne fluoride region indicated extensive bound ultrafilterable fluoride, the total and ionic fluoride being $7.9\ (\pm 0.1) \times 10^{-6}$ mol dm^{-3} and $7.9\ (\pm 0.1) \times 10^{-7}$ mol dm^{-3}, respectively [169]. Jardillier and Desmet [170] found no evidence for

protein-bound fluoride utilising chromatography, gel-permeation or ultrafiltration techniques in human sera from populations in Amiens or the Kaolack region of the Senegal Republic.

Despite this bound fluoride problem, the utility of the fluoride electrode for total plasma fluoride after direct diffusion is illustrated by fluoride analysis of unashed plasma of two rat groups reared on different fluoride intakes [161]. The plasma of rats raised from three weeks old on low fluoride diet (0.5 mg kg^{-1}) and distilled water contained apparent fluoride levels of 4.2 ($\pm$0.8) X 10^{-6} and 3.7 ($\pm$0.4) X 10^{-6} mol dm^{-3} at five and eight weeks, respectively. The corresponding levels in another similar batch, except for supplementation of drinking water to 25 mg kg^{-1} fluoride, were 6.8 ($\pm$0.7) X 10^{-6} and 12.1 ($\pm$0.5) X 10^{-6} mol dm^{-3} fluoride. When the fluoride supplements were withdrawn, the plasma fluoride declined [161].

Apart from plasma fluoride levels and clearance rates in relation to renal transplant patients [171], home dialysis patients [168,172] and inorganic fluoride poisoning cases [173,174] are interesting areas of application of the fluoride electrode. An 18-year-old sodium fluoride-poisoned patient had a blood fluoride level of 0.47 mmol dm^{-3}, the highest-ever recorded fluoride level in blood and a case in which haemodialysis worked well [174].

In an investigation of the movement of fluoride in haemodialysis it has been shown [175] that when the dialysate is prepared with fluoridated water, fluoride moves from the dialysate to blood at a rate comparable with the movement of solutes in the opposite direction. It is suggested that the resulting serum fluoride values are likely to result in altered bone formation [175].

Most major and widely-used inhalant anaesthetics are regarded as safe or safer than general opiods and relaxant anaesthetic [176], but ever since Pringle, Maunsell and Pringle [177] and Thompson [178] in 1905 reported that diethyl ether and chloroform anaesthesia reduced urine output closer attention has been paid to the effect of all anaesthetics. Hence, an important application of the fluoride ion-selective electrode combines fluoride levels in blood and other fluids in connection with the administration of fluorine-containing inhalant anaesthetics. The wide range of such studies merits the detailed discussion in the section following the non-blood fluids.

Miscellaneous ion-selective electrode studies

A knowledge of blood bromide levels is occasionally important as in patients treated with bromosedatives, or anaesthetised with halothane or suffering from drug abuse. The normal bromide level in serum is less than 0.1 mmol dm^{-3} but in cases of bromide intoxication can rise to above 40 mmol dm^{-3} and both levels are measurable by the bromide ion-selective electrode as long as the bromine is

present as bromide anion. However, chloride and cysteine in plasma and urine can interfere and elevated bromide ion concentrations can only be detected when present, say, in plasma at above 1 mmol dm^{-3} [179].

Bromide ion-selective electrode analysis of serum samples taken from an attempted suicide with carbromal after 3 and 24 h gave 0.6 and 1.6 mmol dm^{-3}, respectively, but although more than 3 h must elapse before any diagnosis, such analyses could provide a suitable detection method for ward or outpatient use [179]. A similar instance relates to an 80-year-old patient suffering from bromism following ingestion of tablets containing bromodiethylacetyl urea and α-bromoisovaleryl urea and whose serum bromide level was about 10 mmol dm^{-3} [156].

Another interesting result is that the plasma bromide levels of healthy rabbits remain approximately constant for 12 h after sodium bromide injections [180]. Because of some uncertainty in obtaining an exact bromide ion level from a single millivolt measurement, a method based on a quadratic expression describing a relationship between millivolt readings, E, and bromide ion concentrations was employed in this study [180]:

$$[Br^-]_i = a_0 + a_1 E_i + a_2 E_i^2 \qquad (17)$$

a_0, a_1 and a_2 are constants determined by additions (i) of bromide ions to a blood sample taken before bromide administration, the coefficient of variation for the range investigated was approximately 1 per cent.

Because of irreproducible readings, the Orion 94-82 solid-state lead ion-selective electrode was unsatisfactory for blood and saliva samples [181] but the difficulties concern detection limits for the expected lead levels are less than 1 μmol dm^{-3}.

Serum assays for carbonate may be performed with both a carbon dioxide gas electrode [182,183] and a carbonate ion-selective electrode [182] and good correlations ($r = 0.931$) have been obtained [182,184]. Also, the carbonate content of 27 human serum samples analysed with a carbonate ion-selective electrode correlates well with the more established standard colorimetric Auto Analyser method ($r = 0.9974$) but is considered simpler and potentially more useful for routine and low-cost clinical purposes [185].

NON-BLOOD FLUIDS

Urine

Urine, gastric juice, cerebrospinal fluid, saliva and sweat are the principal non-blood fluids suitable for examination by ion-selective electrodes. Urine presents

problems since conditions of pH, sodium levels and ionic strength vary considerably [134]. Consequently, each urine sample needs to be first analysed for sodium, potassium and ammonium and standards prepared to mimic the urine [134]. About 50 per cent of the calcium in urine is ionised, the remainder being soluble calcium complexes of citrate, phosphate, sulphate and oxalate. Unlike serum calcium, urinary ionised calcium ($\approx 50 \pm 8$–9 per cent) seems to show a marked dependence on the total calcium.

Urinary fluoride, as in body fluids in general, is normally low but is frequently elevated in patients during, and after, fluorine-based anaesthesia and in personnel employed in industries involving aluminium, manganese, hydrogen fluoride, explosives, phosphate fertilisers and fluorocarbons. Atmospheric levels exceeding 4–6 ppm demand surveillance of both personnel and working conditions [186]. The fluoride ion-selective electrode provides a ready practical method for both industrial hygienist [186–199] and the clinical anaesthetist [197–246]. Because of variation in ionic strength, standard procedures usually involve 1 : 1 dilution with chloride-free Total Ionic Strength Urine Buffer (TISUB) or Gran's plots. These methods are justified by excellent recovery values [186,198] and by comparison with microdiffusion data [186–188,193, 194,197,198]. However, attention has been drawn [189] to the fluoride levels obtained by direct electrode measurement being lower than those obtained after microdiffusion and treatment with perchloric acid at an elevated temperature. Such differences attributed to some non-ionic fluoride compounds have not been confirmed [191]. The matter merits further attention.

Four healthy young adult males receiving either 0, 1, 3, 5 or 10 mg fluoride by mouth daily over each of 5 successive weeks gave urinary fluoride rates that were virtually a linear function of dose [190]. For the different doses of fluoride, urine fluoride recovery rates varied from 20.0 to 31.3 per cent (mean = 25.3 per cent) [190]. Humans having fluoridated salt (250 mg F kg^{-1}) in their diet appear from their urinary fluoride to have about the same protection from caries, as with water fluoridation [247].

A placental F-barrier is indicated by the mean urine fluoride concentration of new-born boys being 4×10^{-6} mol dm^{-3} compared with 3×10^{-5} mol dm^{-3} in the *post partum* urine of their mothers [247].

Gastric juice

Problems of pH in gastric juice may be overcome by bringing the samples to pH 6.2–7.2 with sodium hydroxide prior to ionised calcium analysis. In this way, calcium ion-selective electrode studies [248] have shown that the appearance of calcium in gastric juice involves both 'secretory' and 'non-parietal' components. A four-component model was developed to account for the observed calcium

concentrations at all levels of secretory activity for both histamine and gastrin [248], the model being based on the assumption that the observed calcium concentration represents the algebraic sum of four distinct fractions, namely, (i) ionised calcium from gastric intestinal (non-parietal) fluid, (ii) soluble, diffusible calcium complexes, CaR, from nonparietal fluid, (iii) calcium originally bound to albumin, CaA, also a non-parietal component, and (iv) calcium bound to, or associated with, pepsin, CaP, representing the secreted calcium component. Ionised calcium in the histamine studies correlated with total calcium to indicate that about 99 per cent of the total calcium was ionised, while for gastrin the calculated regression line was

$$[Ca^{2+}] = 0.02 + 0.80[Ca] \tag{18}$$

The electrode data appeared compatible with the assumed composition of non-parietal calcium, and in pure secretory fluid the calcium is mostly ionised suggesting that if calcium enters the juice physically bound to pepsin, P, it is displaced from the protein molecule by hydrogen in the very acid juice [248]:

$$CaP + H^{+} \rightleftharpoons Ca^{2+} + HP \tag{19}$$

Cerebrospinal fluid and saliva

Cerebrospinal fluid and saliva present no problems in terms of pH, and ion determinations on saliva could benefit from a wider application of combination (sensor and reference) electrodes. Such a device can be developed for calcium now that sensor membranes with the sensing liquid ion-exchanger trapped in a PVC matrix are well established.

To date, outside of dental matters considered later, the main ion-selective electrode interest on saliva has been the use of sodium glass electrodes for monitoring sodium levels in connection with cystic fibrosis screening, the high sodium content of parotid saliva being one characteristic of the disease [249, 250].

Ion-selective electrode sweat tests for cystic fibrosis

Within the biomedical field, the application of chloride ion-selective electrodes has largely been devoted to sweat analysis in relation to cystic fibrosis screening and diagnosis [159,251–271]. The disease, in which there is a malfunction of exocrine glands, can be readily diagnosed from the elevated chloride ion levels in patients' sweat ($\approx$100 mmol dm^{-3}) compared with a 'normal' ($\approx$25 mmol dm^{-3}). Reasonably accurate monitoring of newborns, infants and children may

also be made by a sweat test with a sodium glass electrode, but the more recent direct-reading skin-chloride reference electrode combination assembly is a more convenient way of detecting the one case per approximately 2000 live births.

The electrode sweat-test methods including sodium glass electrodes [253,256, 272,273] used for screening in cystic fibrosis have recently been reviewed [159] and their reliability for sweat analysis has been thoroughly debated [256] at the recent GAP Conference on 'Problems in Sweat Testing'. Most debators felt that the best method presently available for dependable diagnosis was a pilocarpine iontophoresis sweat stimulation and collection stage followed by quantitative laboratory sodium and/or chloride assay; the chloride ion-selective electrode should, according to its conclusion, be reserved for future use after appropriate development. Such a test is too tedious and expensive for screening purposes and the ion-selective electrode method has a worthwhile role, especially since cystic fibrosis is about one half as common as mongolism, about as common as club foot, hip dislocation and cleft lip and ten times more common than phenylketonuria [255].

The Orion solid-state model 96-17 combination chloride electrode complete with a portable pilocarpine iontophoresis sweat stimulation skin ion meter unit is very convenient for low cost, large scale monitoring of new-born populations. The meter is calibrated with 20 and 100 mmol dm^{-3} standards approximating, respectively, to mean normal and mean cystic fibrosis sweat-chloride levels [253]. Both groups of subjects exhibit a wide range of chloride (and, of course, sodium) values. The clinical analyst has little trouble interpreting chloride values below 35 mmol dm^{-3} and above 65 mmol dm^{-3} as normal and cystic fibrosis, respectively, but confusion arises over diagnosis for chloride within the 35–65 mmol dm^{-3} range [252]. It is in such cases that a reference method such as the Gibson and Cooke [274] sweat stimulation and collection by pilocarpine iontophoresis followed by laboratory analysis for sodium and chloride by flame photometer for sodium and chloridometer for chloride is recommended. Alternatively, sweat induction by ureocholine injection followed by assay with a chloridometer has been employed [251].

The chloride ion-selective electrode screening with its 80 per cent detection rate is highly competitive with alternative methods where again the borderline cases can only be properly identified by careful clinical and repeated laboratory observations. It is the individuals who fail to sweat sufficiently that are difficult and demand special attention because of evaporation [265]. Other problems may be appreciated by realising that the chloride ion-selective sweat screening test has three distinct stages, namely, skin site preparation, stimulation of sweating which is the most intricate step and *in situ* chloride assay with the combination electrode pressed to the stimulated site. Likely sources of problems in these stages include:

(i) pressure variations of electrode-skin surface and time of contact;
(ii) chloride contamination either from breakdown of hexachlorophene in skin washing soaps and/or leakage of 4 mol dm^{-3} potassium chloride from the reference electrode section of the combination electrode;
(iii) uncertainty of the skin temperature in relation to electrode pre-calibration;
(iv) inadequate amounts of sweat;
(v) evaporation of sweat leading to false positives; and
(vi) spurious false positives and negatives reported in some work and the failure of even some experienced technicians to get reproducible results.

Considerable progress has been made on several of these fundamental aspects of sweat analysis. For example, satisfactory stimulation of sweating is achieved with 500 mmol dm^{-3} pilocarpine nitrate in the iontophoresis stage using 1 mA current for 3 min, while a new pad to absorb acid and alkaline skin irritants is so satisfactory that a baby asleep at the start of a test will usually remain asleep throughout [265].

Milk

Ion-selective electrodes have a role for monitoring milk both for its calcium content and as a possible vehicle for health fluoridation programmes. The ion-selective electrode method appears to give higher ionic calcium levels ($\approx$2.71 mmol dm^{-3}) than the method of EDTA titration of ion-exchange eluates ($\approx$2.52 mmol dm^{-3}) possibly because of the smaller pH changes in the former case. However, in passing from raw milk to sterilised milk ($\approx$2.27 mmol dm^{-3}) and pasteurised milk ($\approx$2.04 mmol dm^{-3}) there is a fall in the ionic calcium level [275].

Fluoride contents of milks have been studied in relation to fluoride health programmes in infants [276,277]. Breast-fed infants obtain their fluid intake almost entirely from nursing mothers and a knowledge of fluoride in breast milk aids decisions over fluoride supplementation. Simpson and Tuba [276] found that mothers' milk containing 10.5 ± 0.05 μmol dm^{-3} fluoride prior to the fluoridation of Edmonton water (13.7 ± 0.3 μmol dm^{-3}) rose to 25.8 ± 0.16 μmol dm^{-3} after fluoridation of tap water supplies to 52.6 ± 3.1 μmol dm^{-3}. Thus, breast-fed infants in fluoridated water communities probably receive adequate fluoride, otherwise daily supplementation of about 0.25 mg is recommended [276].

In determining fluorine in milk, the protein-bound material may be released by forming insoluble Amido black–milk protein complexes at pH 2.0 and the fluoride then determined following filtrate adjustment of pH with a citrate buffer [278].

Miscellaneous and intracellular fluids

Intracellular and related fluids are difficult to study because of their relatively minute volumes and it is here that microcapillary electrodes can play a special role. In this respect, the volumes of frog skeletal muscle cells and moluscan neurons are typically 0.25 mm^3 and 0.52 mm^3, respectively, compared with about 10^{-5} mm^3 for a frog heart ventricle cell. Glass electrodes can be readily drawn from cation-sensitive glass with functional impaling tips of less than 1 μm. Although mechanically strong for intracellular research, their cation selectivity is poor and only sodium and potassium cations can be detected additional to pH effects. The wider range microelectrodes developed by Walker [279] comprise finely drawn glass tubes containing a sensor liquid ion-exchanger in the open tip. This design greatly extends the range of ions that can be studied, although problems occasionally arise owing to loss of tip sensor material.

Interest in the measurement of intracellular activities centres on transmembrane potentials and on the transport of ions across membranes. For example, lithium salts feature in the treatment of mania, but owing to the analytical problems little has been known of lithium accumulation or about its transport by nerve cell membranes. However, a recently developed microlithium electrode based on a liquid membrane sensor has established [280] an active transport of lithium ions out of *Helix aspersa* pallial ganglia and that at low external levels there is even less intracellular than extracellular lithium ions. It is therefore difficult to appreciate how lithium could function in an intracellular capacity to alter the neuronal properties in lithium psychiatric therapy [280].

A microcapillary version of the potassium liquid ion-exchanger electrode has been used *in situ* for following potassium ion gradients along the proximal convoluted tubule of a rat kidney [281]. The mean tubular fluid to plasma potassium ion concentration ratio falls significantly from 0.89 for the first convolution to 0.81 for the last convolution of the proximal tubule. Such a disproportionate reabsorption of potassium does not support a common cationic pump mechanism whereby ions and water are reabsorbed in the same proportion as they are first delivered as in the case of sodium [281].

Among the miscellaneous applications of ion-selective electrodes are the use of the Orion calcium electrode for studying calcium ion activity in connection with membrane alkalinisation in mitochondria [282] and on mitosis [283–285]. The electrode has also been used to show that protein polysaccharides of cartillage chelate calcium ions very effectively [286]. Electrode studies of calcium binding by chondroitin sulphate are said to be subject to interference by the condroitin sulphate anion [287].

APPLICATIONS IN DENTAL HEALTH AND MINERALISED TISSUES

The use of ion-selective electrodes in dental and mineralised tissue studies is quite extensive [162,190,192,197,288–360] and particular attention has been devoted to fluoride in saliva and enamel.

SALIVA

The level of fluoride in fasting saliva which normally ranges from 0.5 to 2.5 μmol dm^{-3} depends on the sampling time relative to the individual and the quantities of fluoride consumed [190,308,310,355,356]. These trace quantities could be of importance in preventive densitry since dental surfaces are virtually subjected to continuous topical application of fluoride from the salivary glands.

Grøn, McCann and Brudevold [356] found a rapid rise in the fluoride level in human parotid saliva within 5–10 min of swallowing gelatin capsules containing 10 mg of sodium fluoride and which peaked at 13 μmol dm^{-3} after about 40 min. Similar levels of fluoride occur in samples taken simultaneously from submaxillary and parotid glands [356]. When fluoride is administered as sodium fluoride with aluminium, both the rate of increase, and peak levels, of salivary fluoride are depressed compared with sodium fluoride alone. Fluoride increased even more slowly, and peaked lower, when given as monofluorophosphate (or in conjunction with aluminium) than with neat sodium fluoride and could only be detected as ionic fluoride. Hydrolysis of monofluorophosphate, PO_3F^{2-}, before gastrointestinal absorption of fluoride and the formation of poorly observable fluoroaluminate(III) complexes could account for these observations and dietary aluminium may affect efficient gastrointestinal absorption of fluoride [355]. However, Forsman and Ericsson consider there to be little difference between fluoride absorption from ingested fluoride of fluorophosphate since the latter is rapidly hydrolysed by gut phosphatases [192].

During three-hour-long experiments with healthy male dental students involving continuous exogenous salivary stimulation with peppermint, cherry and grape candy drops, salivary fluoride showed no sign of depletion although the levels were lower than in non-stimulated parotid saliva. Moreover, fluoride concentrations did change despite significant variation in the stimulated flow rates [190], which suggests a passive transport mechanism [308]. The fact that fluoride levels in plasma and saliva are of the same order [349] supports such a mechanism.

No differences have been detected in the salivary elimination of fluoride in children with and without kidney disfuction [333].

Tatevossian and Jenkins [357] conclude from studies with a calcium elec-

trode that saccharate formation is unlikely to contribute to the dissolution of enamel in caries.

PLAQUE

The fluoride content of natural or experimental plaque from buccal teeth surface or intra-oral block devices is about 1000 to 2000 times higher than saliva [356] although greater levels have been found in 143 children 11 to 13 years old after regular weekly mouth rinses with 0.2 per cent sodium fluoride over four years [312].

The fluoride which reacts slowly with the plaque is considered to be bound by low molecular weight cations and not carbohydrates, proteins or oral streptococci [317]. Frequent and regular supplies of fluoride are desirable to maintain any anticaries action in plaque [316] where fluoride inhibits acid production and is considered to protect erupted teeth [353].

DENTRIFICES, TABLETS AND MOUTH RINSES

Fluoride in dentrifices is usually present as sodium fluoride or monofluorophosphate or tin(II) fluoride [192,313,341,358,359]. Analysis of aqueous extracts or slurries of such dentrifices take less than 10 min with the fluoride electrode and closely match those obtained by a diffusion–thorium(IV) titration method but which requires about 45 min per sample [358].

Studies with rats, human adults and some children indicate that calcium-based polishing agents in five commercial and four experimental toothpastes prevent gut absorption of a considerable fraction of fluoride and monofluorophosphate owing to complexation [192]. Except for small initial losses of uncomplexed fluoride by interaction with calcium carbonate polishing particles, no major depletion of active ionic fluoride is found to occur in toothpastes over one year [359] but another study indicated considerable losses of free fluoride after only four months of shelf life [341]. It should be noted that analysis with the fluoride electrode might be unreliable in samples containing monofluorophosphate [360], glycerin [313] and silica gel [313].

Caries inhibition resulting from daily administration of chewable tablets or mouth rinses may be due to incorporation of absorbable fluoride into the developing enamel teeth surfaces. The retention of fluoride following oral rinses [311, 315, 336] or tablet chewing [336] has been followed by analysing the fluoride of the oral expectorates. The anti-caries effect of ionic fluoride might then be evaluated in relation to fluoride retention [311]. Perkins found oral retentions of 41, 20 and 7 per cent of the fluoride dose, respectively, administered as

sodium fluoride tablets, acidulated phosphate-fluoride (APF) tablets and aqueous APF rinses [336].

ENAMEL

The fluoride electrode has dramatically extended *in vivo* and *in vitro* studies on the relation between fluoride in tooth enamel and the incidence of dental caries and the incorporation of fluoride from ingested and topical fluorides.

The well-known fluoride gradient decreases very sharply with depth from the enamel tooth surface towards the amelo-dentinal junction [318,350,251]. Minor variations in the thickness of outermost enamel layers can profoundly affect analysis unless proper care is exercised to avoid extraneous contamination. Biopsy techniques are thus difficult since sampling alters teeth surfaces and two identical surface areas do not exist [318]. Abrasion with silicon carbide–glycerol slurries for less than 1 min has proved quite satisfactory for this purpose. Samples of about 40 μg, and equivalent to about 0.3 μm enamel, are either collected in plastic [351] or rubber cups [318], dissolved in 0.5 M perchloric acid and analysed directly with the fluoride electrode. Most teeth except those posteriorly located can be analysed by the abrasion technique.

First biopsies for fluoride in the outer 1–2 μm layers of intact anterior teeth of an individual are similar but vary from 400 to 2500 mg kg^{-1} in a population where about 100 mg kg^{-1} is considered as the minimum requirement for caries prevention. Biopsies taken on the second day or even five weeks later from the same teeth showed much less fluoride [351]. In a study of 23 Danish children the first layer (about 0.5 μm) of teeth contained 2000–5800 mg kg^{-1} fluoride compared with 1500–4800 mg kg^{-1} in the second layer of the same thickness [318].

Thus any biopsy technique must control depth and total enamel removed since a thick layer will be invariably lower in fluoride than a thin layer from the same enamel. A major criticism of this biopsy technique is that the pressure, area and depths of the enamel regions examined are not carefully standardised and an electrically driven abrasive device is considered to facilitate greater control of quantitative removal of surface enamels [320,321].

The procedures resemble dental prophylaxis and biopsies have been conducted on over hundreds of patients without apparent complaint. No more enamel is lost than in pumice treatments and it also gives a highly polished surface and chair-time is brief (about 2 min). Several very thin layers of the *in vivo* enamel can be successively removed from individual teeth under a variety of oral environments. Long-term programmes of fluoride–enamel interactions can thus be very conveniently studied.

A number of chemical agents other than NaF, Ha_2PO_3F and SnF_2 have been

examined because it is important to establish which is the most effective anticaries agency. Titanium(IV) fluoride is considered to react with enamel and increase its fluoride content and decrease its solubility [297]. Unfortunately, such fluoride will leach from enamel surface unless the topical treatment is continued. The application of fluoride-releasing polyurethane coatings to enamel could prove a most beneficial long term anticaries measure. *In vitro* studies with animal [332] and human [345] teeth have established a reduction in acid solubility of enamel [332] and a retention of about 2000 mg kg^{-1} fluoride in the outer surfaces at least after one week of washing [345]. In the *in vivo* programme, the single application of lacquer releasing fluoride effectively reduced carious lesions of albino rat molars fed on higher sugar diets and inocculated with a cariogenic bacterium [332]. Such fluoride-release coatings might be used for all surfaces of teeth [332].

Several interesting *in vivo* studies have concerned the concentration of fluoride in the outermost tooth layers of hamsters [303] and rats [301,304,305] following long-term administration of citrous and cola beverages with and/or without fluoride supplementation. The erosive effect of excessive consumption of fruit juices on enamel in these experimental animals is decreased by fluoride supplementation. Moreover, the mean fluoride content of molar surface gradually increased with increased exposure times being greater for mandibular than maxillary teeth, possibly due to the lower molars being in more frequent contact with oral fluids. The well-known etching of these acid beverages possibly exposed inter-crystalline spaces in the enamel and thereby enhanced fluoride uptake [305].

The mechanism by which fluoride ions inhibit caries is not fully understood [361]. One mechanism involves substitution reactions in the surface hydroxyapatite of enamel to create a concentration gradient of less acid-soluble, but isostructural, fluoroapatite (fluoride content about 38 000 mg kg^{-1}):

$$Ca_5(PO_4)_3OH(s) + H_3O^+ + F^- \rightleftharpoons Ca_5(PO_4)_3F(s) + 2\,H_2O;\ \Delta H^0 = -96\ kJ\ mol^{-1}$$

although X-ray and infrared studies show that calcium fluoride (fluoride content about 490 000 mg kg^{-1}) is also formed but to an unknown extent [328]:

$$2\,Ca_5(PO_4)_3OH(s) + 2\,HF \rightleftharpoons 3\,Ca_3(PO_4)_2(s) + CaF_2(s) + 2\,H_2O;$$

$$\Delta H^0 = -12\ kJ\ mol^{-1}$$

The fluoride found in the outer 1–2 μm of human enamel within 2 h of topical fluoride treatment is usually in the range 30 000–35 000 mg kg^{-1}, occasionally 35 000–40 000 mg kg^{-1} but never more [330]. Stearns thus proposes fluoroapatite to be the dominant surface enamel species immediately

following fluoride application because adhering calcium fluoride would boost the enamel fluoride well above the 38 000 mg kg^{-1} mark. The calcium fluoride detected is considered to be easily sloughed from such enamel surfaces [330]. In any event, the surface fluoride falls rapidly from around 38 000 mg kg^{-1} to 13 000 mg kg^{-1} and 8100 mg kg^{-1} within 7 and 14 days of post-topical fluoride treatment [329] (see p. 104).

BONE

The good agreement found between fluoride in rat bones using an electrode and diffusion–colorimetric methods established the prospective utility of the fluoride ion-selective electrode. After dissolution in hydrochloric acid and pH-ionic strength adjustment, the potentials of ashed bone samples are related to fluoride content from calibration standards [289].

An autopsy survey of ribs and vertebrae of two groups of cadavers has confirmed that mature bones acquire fluoride and its concentration relates to water fluoride levels. The incidence and severity of intervertebral osteoarthritis and osteoporosis seemed unaffected by the fluoride in potable waters at least over a 5–50 μmol dm^{-3} range [290]. The proportionate rates of increase of fluoride in plasma and bone with age and the similarity of the correlation coefficients imply an equilibrium between bone fluoride and plasma fluoride [337].

ION-SELECTIVE ELECTRODE STUDIES OF ANAESTHETIC METABOLISM

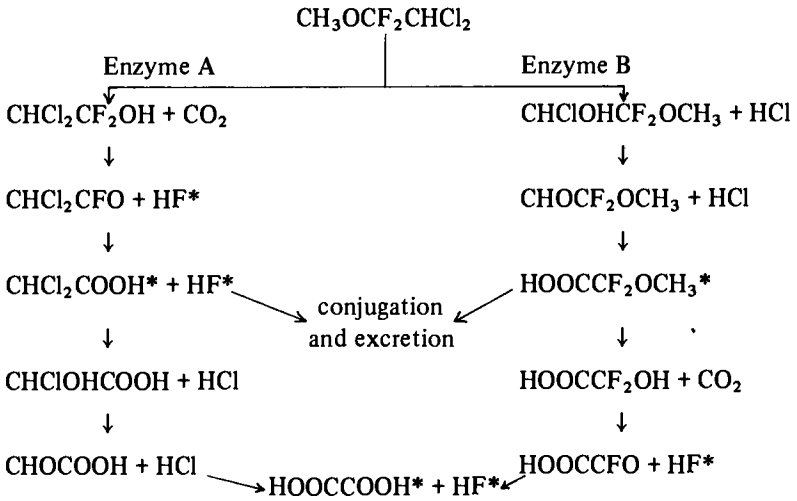
The use of inhalational and parenteral anaesthetics is aimed to cause minimal adverse effects. Generally, anaesthetics administered judiciously and even in moderate overdose have not been found to cause a direct adverse effect on the renal parenchyma in animals or man unless they accompanied or followed severe surgical trauma and multiple blood transfusions [230]. Nevertheless, there is usually a reduction in urine output during surgical anaesthesia and associated reductions in renal blood flow, glomerular filtration rate and osmolar clearance but such effects usually respond to forced diuresis with intravenous fluids [230]. Although effects may generally be minimal, the fact that they exist together with consciousness of the toxicity dangers leading to renal, hepatic and hepato-renal failures, give emphasis to an understanding of the biotransformations that may occur. This need has been brought into even greater prominence since 1960 by the introduction of a series of fluorinated methyl ethyl ethers and similar materials into clinical practice (*Table 2.4*).

Renal failure has been reported following human administration of methoxy-

Table 2.4. SOME FLUORINATED VOLATILE ORGANIC ANAESTHETIC DRUGS

Non-proprietary name	*Chemical formula*	*Chemical name*
Methoxyflurane	$MeOCF_2CHCl_2$	2,2-dichloro-1,1-difluoroethyl methyl ether
Isoflurane	$CHF_2OCHClCF_3$	2,2,2-trifluoro-1-chloroethyl difluoromethyl ether
Enflurane	CHF_2OCF_2CHFCl	2-chloro-1,1,2-trifluoroethyl difluoromethyl ether
Sevoflurane	$CH_2FOCH(CF_3)_2$	1,1,1,3,3,3-hexafluoroisopropyl fluoromethyl ether
Fluroxene	$CF_3CH_2OCH{=}CH_2$	2,2,2-trifluoroethyl vinyl ether

flurane as an anaesthetic [238] and the effect is dose-related [216], the severity of disfunction being proportional to serum inorganic fluoride [218]. Less than 50 per cent of the chemical is exhaled [237], the remainder appearing as serum and urinary metabolites, among which is ionic fluoride considered to be probably the nephrotoxin [217]. Urinary products detected include oxalic acid, dichloroacetic acid, 2,2-difluoromethoxyacetic acid and inorganic fluoride wherein both the bound urinary 2,2-difluoromethoxyacetic acid (after Schöniger oxygen flask combustion) and the free fluoride ion are conveniently evaluated with the fluoride electrode. Suggested metabolic pathways are [207,224] shown in *Scheme 2.1.*



Scheme 2.1. Metabolic pathways for methoxyflurane. Detectable urinary products are denoted by an asterisk

The actual level of fluoride in the sera of anaesthetised patients with methoxyflurane or fluroxene [209] depend on preoperative drug medication and supplementary anaesthetic such as fetanyl–nitrous oxide. The degrees of obesity [208] and length of operations [216] are also contributory factors.

Non-obese patients (45–81 kg) anesthetised with methoxyflurane and nitrous oxide (40 : 60) for 3 h (17 patients) and 5 h (11 patients) and maintained thereafter with fentanyl–nitrous oxide had the inorganic fluoride in their venous blood determined before induction, after 1 and 3 h of anaesthesia and after 2, 24, 48 and 72 h following conclusion of anaesthesia [209]. In each group, the maximum fluoride levels occurred 24 h after the operation, being greatest for 5 h patients (67.2 ± 8.8 μmol dm^{-3} vs. 43.9 ± 5.7 μmol dm^{-3}) but the levels in both sets of serum fluoride levels of 19 chronically obese adults (153 ± 9.6 kg) anaesthetised for 3 h reached 55.8 ± 5.8 μmol dm^{-3} in 2 h after discontinuing administering the methoxyflurane–nitrous oxide (40 : 60) mixture [208]. These fluoride levels increased more rapidly, peaked higher and sooner compared with non-obese patients (71.9 ± 4.9 kg) which peaked at 43.9 μmol dm^{-3} after 24 h [209]. Liver biopsies showed moderate to severe fatty infiltration, thereby possibly increasing uptake of methoxyflurane and exposed more methoxyflurane to hepatic microsomal enzymes [208]. Control halothane–nitrous oxide anaesthesia with 12 obese patients led to a peaking in the serum fluoride level after 3 h anaesthesia (10.4 ± 1.5 μmol dm^{-3}) [208].

A separate study with patients anaesthetised without nitrous oxide supplementation gave peak serum fluoride values of 190.4 ± 20.9 μmol dm^{-3}, probably owing to the higher dose of methoxyflurane [218].

Fluroxene also undergoes biotransformation in man and a suggested metabolic pathway passes through trifluoroethanol and trifluoroacetaldehyde to trifluoroacetic acid [235]. Ten per cent of the amount administered has been recovered mainly as urinary trifluoroacetic acid while the highly toxic trifluoroethanol accounted for only 0.6 per cent of the total fluorene administered [234]. Serum ionic fluoride in 5 patients (normal fluoride 1.0 ± 0.1 μmol dm^{-3}) never exceeded 2.9 ± 0.6 μmol dm^{-3} for 24 h post-operatively following fluroxene–nitrous oxide (40 : 60) anaesthesia [209]. Here it is relevant that a quantitative autopsy of liver, adrenal and adipose tissue of a 47-year-old Caucasian female showed trifluoroacetic acid as the principal metabolite of fluroxene [200]. Nevertheless, there are several reported instances of toxicity following fluroxene anaesthesia in man but with associated histories of medication with such microsomal enzyme-inducing drugs as phenobarbital and phenytoin and pointing to trifluoroethanol being the offending agent [235]. Although fluroxene has been used for about 20 years with about half a million patients, Ohio Medical Products have discontinued its manufacture for clinical use [235].

The toxicity of trifluoroethanol is about 12 times that of trifluoroacetic acid

which accounts for fluroxene being consistently toxic to animals such as mice, dogs, cats and rabbits whose metabolic pathways favour trifluoroethanol formation. Here the metabolic pathways of the rhesus monkey resemble those of man more closely and give tolerance to fluroxene exposure for up to 16 h [236].

Isoflurane is more resistant to biotransformation in man than are other volatile halogenated anaesthetics [201,241,245] although there can be metabolic variations in different animal species [245]. An average recovery of 95 per cent has been obtained in exhaled air, while post-operative urinary excretion of ionic and bound organic fluoride accounted for less than 0.2 per cent of the total fluorine dose [201]. In a separate study of 9 surgical patients, serum inorganic fluoride levels were 4.4 ± 0.4 μmol dm^{-3} 6 h after anaesthesia [241].

Biotransformation of enflurane has been followed by measuring urinary excretion in 7 healthy females [219]. Only 0.5 per cent and 1.9 per cent were recovered as urinary inorganic and organic fluoride, respectively, and which peaked after 7 h and two days, respectively. Exhaled enflurane accounted for 82.7 ± 18.8 per cent of the total dose [219]. The peak values of serum inorganic fluoride in 102 patients administered enflurane rarely exceeded 50 μmol dm^{-3} and in this study did not last for the long periods so characteristic of methoxyflurane [246].

Sevoflurane administered to groups of Fischer 344 rats yielded one third to one fourth the urinary inorganic fluoride associated with similar levels of methoxyflurane and no renal functional or morphologic defects were observed following administration of the anaesthetic for up to 10 h. However, there was an unexplained significant weight loss which occurred following all exposures [244].

Associated with the above studies are those on fluoroethylenes, and hexafluoropropene [228] and on perfluorobutyl perfluorotetrahydrofuran (FX-80, a liquid oxygen-transport medium) [202]. The increase in the urinary fluoride following various exposures in the former case suggest a degradation to inorganic fluoride [228] but no excess fluoride ion was found in the urine of 17 dogs following liquid breathing with oxygenated FX-80 [202]. The FX-80 is distributed in the body according to the lipid content of tissues and according to calculations a 13.8 kg dog would absorb 1.25 g of FX-80.

APPLICATIONS OF ENZYME SYSTEMS AND ELECTRODES

Enzyme electrodes are modified conventional ion-selective electrodes [362] wherein an enzyme- or substrate-soaked matrix is sandwiched in a gel layer between the sample solution and sensing membrane of the electrode [363–370]. They are covered by the definition [371] that 'Enzyme–substrate electrodes are

sensors in which an ion-selective electrode is covered with a coating that contains an enzyme which causes the reaction of an organic or inorganic (substrate) to produce a species to which the electrode responds. Alternatively, the sensor could be covered with a layer of substrate which reacts with the enzyme to be assayed'.

The functional principles of these electrodes are illustrated by a Beckman univalent electrode (Model 39137) coated with urease. This electrode when brought into contact with urea solution [364] produced NH_4^+ cations:

$$CO(NH_2)_2 + 2\,H_2O \xrightarrow{\text{urease}} 2\,NH_4^+ + CO_3^{2-} \qquad (20)$$

which diffuse through the gel layer to the glass sensor surface and there produce a potential in the usual manner. Factors such as gel thickness, enzyme concentration and the method of enzyme–gel matrix preparation all affect the quality of the ion-selective electrode [368].

Possible applications of enzyme electrodes include the determination of urea in blood (Equation (20)) and for which the optimum pH is about 7 at which 99.7 per cent of the ammonia product exists as NH_4^+ cation, the only form among the species generated that elicits a potential response from the Beckman cation-selective glass electrode. Any sodium or potassium ion interference introduced with the enzyme (or substrate) can easily be detected because of the large positive potential reading that it causes. In such instances Dowex 50 cation exchanger may be used to remove the interferences beforehand [372]. Average differences between the urea values in blood and urine as determined by the urea–urease electrode and a spectrophotometric technique were 2.8 and 2.3 per cent m/m, respectively [373].

Blood and serum sodium and potassium interferences may also be overcome by using ion-selective electrodes with better cation selectivity [374–377]. Thus, a silicone rubber matrix containing nonactin ($k^{pot}_{NH_4K} \approx 1.5 \times 10^{-3}$ and $k^{pot}_{NH_4Na} \approx 1.2 \times 10^{-3}$) and coated with urease–polyacrylamide gel provides [366] a long-life ammonium ion sensor with about a 2 mV drift over 5 weeks and time responses of about 1 min. Again, good agreement with spectrophotometry is obtained for the assay of urea in serum [375].

As implied, the optimum pH level of about 7 for deaminase fixes the ammonia product in the form of the ammonium cation. Raising the pH value to about 12 shifts the equilibrium to favour ammonia gas and for which there are available sensing electrodes like the flow-through Orion 95-10 combination ammonia gas electrode. However, most enzymes are inactivated at such high pH, but Llenado and Rechnitz [377] solved the equilibrium problem by first completing serum urea–urease incubations at the optimum pH of 7.4 in a static stage lasting for 20 min at 37°C. Samples were then bled off, the pH raised to 12 by quenching

with 0.5 mol dm^{-3} sodium hydroxide solution and the ammonia gas measured with an ammonia gas electrode. The potentials displayed on the associated recorder were proportional to ammonia concentration and the urea concentration is one half of this (Equation (20)).

In principle, any metabolic process that terminates in the production of ammonia or carbon dioxide can be similarly quantified in a continuous, automatic, incubation-quenching technique. Already some progress has been made with creatinine, another nitrogenous terminal metabolite, using an ammonia gas electrode [376], while glutaminase activity (arising from isoenzymes present in rat tissues and tumours) may be similarly determined [378]. Urea and tyrosine can be assayed [367] by using the appropriate decarboxylase and a carbon dioxide gas electrode.

The air-gap electrode [150] may be used in the secondary quantitative stage just as conveniently as other designs of gas electrode and with the added advantage that the ion-selective electrode sensing surface does not make contact with the fouling proteins present in many clinical samples. The rapid determination of urea in blood over the range 10^{-2}–10^{-4} mol dm^{-3} has been reported for this electrode [150] and values were within ±2.2 per cent of AutoAnalyzer values [379]. The excellent stability of the air-gap electrode facilitated the assay of 460 samples over 4 weeks, the cost per assay being just 2 per cent of that of the AutoAnalyzer method, but the response time per sample rose from an initial 3 min to about 5–6 min over the period [379].

The many instances of enzyme-based assays for amino acid systems have been recently reviewed [362]. On the wider front may be cited the assay of acetylcholinesterase which converts acetylcholine (ACh) into choline (Ch) and followed with a Corning 476200 liquid-membrane acetylcholine bromide-selective electrode [380,381] for which $k^{pot}_{AChCh} \approx 0.06$. An improved version of the electrode has been constructed from a PVC-acetylcholine-tetra-*p*-chlorophenylborate membrane including phthalate plasticiser [382].

The assay of serum cholinesterase has been based on the measurement with a Corning glass pH electrode of the acetic acid produced from acetylcholine with a precision of 1.3–3.5 per cent [383]. The hydrolysis reaction

$$Me_3N^+CH_2CH_2SCOMe \xrightarrow{\text{cholinesterase}} Me_3N^+CH_2CH_2SH + MeCOOH \qquad (21)$$

can also be used in a flowing system with a sulphide ion-selective electrode responding to the thiocholine product [384].

The above enzyme systems depend on the availability of ion-selective electrodes that are compatible with the substrate and/or the product. However, there are many enzyme systems of the type

$$\beta\text{-D-glucose} + H_2O + O_2 \xrightarrow{\text{glucose oxidase}} \text{D-gluconic acid} + H_2O_2 \qquad (22)$$

where neither substrate nor product can be directly detected by ion-selective electrodes. Nevertheless, the consumption of iodide by the hydrogen peroxide product at pH 5.1:

$$H_2O_2 + 2\,H_3O^+ + 2\,I^- \xrightarrow{\text{Mo(VI) catalyst}} I_2 + 4\,H_2O \qquad (23)$$

can be monitored with the Orion 94-53A iodide ion-selective electrode to provide an excellent indirect assay of that product [385]. Up to 40 glucose samples per hour can be processed by this method.

The same reaction has been studied in a flow system coupled with a sensor-reference pair of iodide ion-selective electrodes but with the more efficient peroxidase catalyst substituted for molybdenum(VI) [386]. Maltose and cellobiose interfere while samples containing the more serious ascorbic acid, tyrosine or uric acid interferents require pre-treatment [386].

MEDICINAL COMPOUNDS AND MATERIALS

Bromine, chlorine, cyanide, iodine and particularly fluorine and sulphur have each been determined in monitoring a wide range of organic compounds with ion-selective electrodes. The general principles apply to compounds of medicinal and pharmaceutical interest. Hot flask combustion [387,388], oxygen flask combustion [389–392], and fluorine liberation with sodium biphenyl reagent [393] have each been used to prepare samples of such compounds for fluorine analysis. The subsequent stages involve pH adjustment with TISAB or otherwise, regardless of whether the final determination is direct [388,391] or by potentiometric titration [387,389,390,393].

Although the subsequent stages are handled in polythene or PTFE vessels, the combustion stage is carried out in glass vessels [387–390]; Shearer and Morris [391] modified a polypropylene sample bottle, while Hozumi and Akimoto [392] used a silica flask to avoid low results obtained with borosilicate glass flasks. What is important is that boosters such as decanol or benzoic acid should be present for supplying sufficient hydrogen to form hydrogen fluoride with fluorine-rich compounds [387,391,393,394]. Without this precaution, carbon tetrafluoride (not detected by the fluoride ion-selective electrode) has been found in the infrared spectra of combustion products of highly fluorinated materials [387] although Selig [390] has reported excellent fluorine recoveries without a combustion booster using Vycor glass flasks even for Teflon.

Fluorinated groups are found in several medicinal and agricultural chemicals, including diuretic, antiemetic and antihypertensive agents, and pre-emergent herbicides. This emphasises the need for simple analytical methods for monitor-

ing and for describing the mode of physiological disposition and mode of action of pharmacologically active compounds. The above methods are convenient for solid and liquid samples. Compounds with labile carbon–fluorine bonds may be monitored following hydrolysis of the bonds with alkali to release fluoride which may be determined with the fluoride ion-selective electrode. In this way, the differential hydrolysis rates of the carbon–fluorine bonds in 5-trifluoromethyl-2′-deoxyuridine, a potential cancer chemotherapeutic agent, and its metabolite, 5-trifluoromethyluracil, permit their determination in urine [395].

Organic compounds of pharmaceutical and pesticidal interest have been analysed for other halides [396–404]. Thus, the ionisable chloride of hydrochlorides of drugs like tetracycline hydrochloride, pethidine hydrochloride and thiamine hydrochloride can be simply dissolved in water and assayed directly or by potentiometric titration [396,397]. Bound halides as in myelbromol, chloramphenicol and iodochlorhydroxyquinoline require a preliminary Schöniger oxygen-flask stage prior to their potentiometric titration with an appropriate halide ion-sensitive electrode [397,398,400]. Iodine liberated from the hormones, 3,3′,5′-triiodo-L-thyronine and 3,5,3′,5′-tetraiodo-L-thyronine by treatment of sample suspensions at pH 11 with nascent hydrogen has been determined with an Orion iodide ion-selective electrode following neutralisation with hydrochloric acid [404].

The chloride content of pharmaceutical-grade aluminium hydroxide gels has been determined with a chloride ion-selective electrode on an aqueous suspension of the material [405].

The quantitative reduction, or photodecomposition by illumination with a powerful light source, of cyanocobalamin liberates hydrogen cyanide, and this may be used to precede the assay stage in pharmaceutical preparations [406] with a cyanide ion-selective electrode at pH 11.

Sulphur can be determined in sulphur-containing compounds with a lead ion-selective electrode by potentiometric titration after oxygen-flask combustion to sulphate [407], but any phosphate which forms a very insoluble lead salt must first be removed. The recently developed barium ion-selective electrode is also convenient for this purpose [408].

Many sulphur compounds can be assayed without prior combustion owing to certain subtle properties of organic sulphur bonds [409–415]. Thus, thiourea [410] can be assayed directly in sodium hydroxide media with a sulphide ion-selective electrode. The assay is supported by reactions (24) and (25):

$$(NH_2)_2CS + 2\ AgNO_3 \rightarrow NH_2CN + 2\ HNO_3 + Ag_2S \qquad (24)$$

$$NH_2CN + 2\ AgNO_3 \rightarrow Ag_2NCN + 2\ HNO_3 \qquad (25)$$

whose stoichiometries are supported by infrared and elemental analysis of

products. The lower limit is 10^{-6} mol dm^{-3} by direct potentiometry and 10^{-3} mol dm^{-3} by titration [410]. Thiouracils [411], phenylthiourea [409] and *N,N*-diphenylthiourea [409] can also be simply determined with silver nitrate. Selig [414] has similarly analysed many thiols with mercuric perchlorate and a bromide ion-selective electrode except for certain thiols with heterocyclic rings.

Simple thiols like L-cysteine, 2-mercaptoethanol and glutathione have also been titrated [412] and the –SH thiol group content agreed to within ±2 per cent of those by an alternative iodometric technique. The procedure has been extended [413] to proteins following a preliminary two-stage reduction of disulphide –S–S– groups to the titratable thio form. Except for lysozyme, values for –S–S– groups in compounds like trypsin, ribonuclease, β-lactoglobulin, α-chymotrypsin, insulin and serum albumin came to within 7 per cent of literature values [413].

Certain food and drug regulations regard all slow-release preparations as drugs and require their safety to be established prior to clinical application. Thus, a potassium ion-selective electrode has been employed to follow [416] the release parameters of 12 encapsulated potassium chloride tablets. Several of the tablets showed only medium dissolution rates for potassium and are unlikely to cause adverse effects such as gut ulceration. A sodium electrode has been similarly used to study the release of sodium phenobarbitone through a dialysis membrane into tris buffer [417]. Sodium in organic compounds has also been determined with an Orion 94-11 sodium electrode following combustion in a closed flask [418].

The increased use of sodium-based infusion and injection fluids in clinical medicine and the introduction of intermittent interperitonial dialysis and haemodialysis has stimulated interest in the idea of using the presence of the sodium common factor as the basis for analytical control. Pearson and Gray [419] have used the EIL GEA33 sodium glass electrode to explore this idea and summarise products that may have their sodium content measured directly without dilution and suggest dilutions with Tris buffer or water as appropriate for certain more complex products containing sodium salts of weak acids, mixtures containing electrolytes other than sodium chloride and dextrose, and haemodialysis concentrates. The direct potentiometric method gave a mean sodium content for 50 samples of 'Sodium Chloride Injection BP' (containing 150 mmol dm^{-3}) of 153.8 mmol dm^{-3} compared with a Mohr titration mean of 153.2 mmol dm^{-3} [419]. Such data are to be seen in the light of the official BPC limit of ±5 per cent of the stated sodium concentration although certain hospitals such as Aberdeen Royal Infirmary (±4 per cent) and Newcastle General Hospital (±3 per cent) have been working to tighter limits [419].

COMPLEXES OF BIOMEDICAL SIGNIFICANCE

Because of the ability of ion-selective electrodes to measure the activity of free ions, it has been possible to obtain thermodynamic formation constants and to have information on the number of binding sites and the stoichiometries of complexes. Thus, formation constants have been calculated for copper(II) complexes with glycine, glutamic acid and tris(hydroxymethyl)aminomethane [420,421].

Saturated solutions of calcium carbonate under atmospheric carbon dioxide have been shown to contain 80 per cent Ca^{2+} with the other 20 per cent dissolved calcium being $CaCO_3$ and $CaHCO_3^+$ species [422]. Of rather more significance in the dental and mineralised tissue field is the complexation of calcium ions with various inorganic phosphates where results for tetrapolyphosphates, trimetaphosphate, and tetrametaphosphate obtained with calcium ion-selective electrodes match those of pH titrations [423,424].

The emf difference, ΔE, between cells of the type

$$\text{Cation-selective electrode} \left| \begin{matrix} MCl_x \\ m_1 \end{matrix} \right| \text{AgCl, Ag}$$

and

$$\text{Cation-selective electrode} \left| \begin{matrix} MCl_x & \text{amino acid (aq)} \\ m_1 & m_2 \end{matrix} \right| \text{AgCl, Ag}$$

have been used to compare activity coefficients of sodium in the presence and absence of glycine [425], while for calcium chloride when ΔE is given by

$$\Delta E = \frac{3RT}{2F} \ln \frac{f_{\pm}^0}{f_{\pm}} \tag{26}$$

where $f_{\pm}^0$ and $f_{\pm}$ are the mean ionic activity coefficients of calcium chloride in the absence and presence of amino acid, respectively, salting-in parameters for amino acids by calcium chloride have been calculated [426]. For glycine, β-alanine, γ-aminobutyric acid, ϵ-aminocaproic acid and glycylglycine, such parameters range from 0.759 to 1.663 mol^{-1} kg [426].

Ion-binding studies with biological molecules can be important in elucidating fundamental biochemical reaction systems in relation to bioenergetics, enzyme activation and membrane transport [182]. For example, the adenosine triphosphate (ATP)–adenosine diphosphate (ADP) cycle is one of the processes of primary importance to cellular energy systems and association constants determined [427–430] for metal–ATP and metal–ADP complexes are therefore of considerable interest (*Table 2.5*). The constants may be obtained from measure-

Table 2.5. ASSOCIATION CONSTANTS OF ATP AND ADP COMPLEXES MEASURED WITH ION-SELECTIVE ELECTRODES AT 25°C AND ZERO IONIC STRENGTH [182,430]

Reaction	*Association constant* ($dm^3\ mol^{-1}$)	*Electrode used*
$Na^+ + ATP^{4-} \rightleftharpoons NaATP^{3-}$	2.29×10^2	Glass
$K^+ + ADP^{3-} \rightleftharpoons KADP^{2-}$	1.50×10^2	Valinomycin-based liquid membrane
$Ca^{2+} + ADP^{3-} \rightleftharpoons CaADP^-$	2.87×10^4	Liquid membrane Ca^{2+}
$Ca^{2+} + CaADP^- \rightleftharpoons Ca_2ADP^+$	2.70×10^2	
$Mg^{2+} + ADP^{3-} \rightleftharpoons MgADP^-$	4.42×10^4	Divalent liquid membrane
$Ca^{2+} + ATP^{4-} \rightleftharpoons CaATP^{2-}$	2.34×10^6	Liquid membrane Ca^{2+}
$Ca^{2+} + CaATP^{2-} \rightleftharpoons Ca_2ATP$	1.10×10^3	
$Mg^{2+} + ATP^{4-} \rightleftharpoons MgATP^{2-}$	1.15×10^6	Divalent liquid membrane
$Mg^{2+} + MgATP^{2-} \rightleftharpoons Mg_2ATP$	4.05×10^2	
$K^+ + ATP^{4-} \rightleftharpoons KATP^{3-}$	2.29×10^2	Valinomycin-based liquid membrane

ments with appropriate ion-selective electrodes and values of approx. 2.3×10^2 $dm^3\ mol^{-1}$ for $KATP^{3-}$ and $NaATP^{3-}$ are substantially greater than those of approx. 10–15 $dm^3\ mol^{-1}$ previously obtained from indirect measurements [427,428].

Calcium electrode measurements with the calcium ATP system have shown the existence of Ca_2ATP ($K_2 = 1.10 \times 10^3\ dm^3\ mol^{-1}$) in addition to the previously established $CaATP^{2-}$ complex whose 'electrode' value for K_1 was $2.34 \times 10^6\ dm^3\ mol^{-1}$ compared with 2.0×10^3 by pH filtration and 3.2×10^4 by spectrophotometry [429].

The importance of data such as those in *Table 2.5* lie in the effect of metal ion activities along with pH, ligand concentrations and other variables on the energy released by ATP hydrolysis:

$$ATP + H_2O \rightleftharpoons ADP + P_i \qquad (27)$$

where P_i represents inorganic phosphate [182]. Such effects can cause the free energy of hydrolysis to vary by several thousand joules with changing conditions. Arising from such studies, Rechnitz [182] has demonstrated that the presence of the newly discovered 2 : 1 complexes for alkaline earth cations with some of the nucleotides in equilibrium with 1 : 1 complexes has a significant effect in main-

taining the free energy of ATP hydrolysis fairly constant over a wide range of metal ion levels. On the other hand, 1 : 1 complexes alone suggest a continuing change of free energy in the direction of increasing spontaneity such that organisms deriving energy via ATP hydrolysis would benefit from internal environments having very high metal ion levels, but organisms have not been found to show such dependence.

Spectrin, a protein located at the inner surface of the red blood cell membrane, has been shown to bind to calcium ions and the feature may be relevant to the effect of calcium in changing the shape of red cell membranes [431,432].

CONCLUSION

Although ion-selective electrodes have now been used for about a decade, compared with the longer record for glass electrodes, they have made many practical contributions in the biomedical field and created new areas of interest.

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Note added in proof

p. 82. These conclusions [329] need re-examination in the light of recent studies of enamel surfaces pretreated with an acid phosphate–fluoride gel and examined by X-ray photoelectron spectroscopy which revealed a surface material devoid of phosphorus and comprising calcium fluoride [433]. Removal of further successive layers by Auger etching (approx. 3 nm h^{-1}) established a steady decrease in fluoride content but a corresponding rise in phosphorus.

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3 Biological Activity of Polyene Antibiotics

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INTRODUCTION

Macrolide antibiotics, produced almost exclusively by *Streptomyces*, are characterised by the possession of a macrocyclic lactone ring containing one or more sugar residues. This class of antibiotics is divided into two groups with contrasting antimicrobial activities. The first group as typified by erythromycin, shows activity against bacterial protein synthesis. The macrolide ring of the second group is larger and contains a series of conjugated double bonds, which gives the group its name, the polyenes. This unsaturation markedly changes the antimicrobial properties of the macrolide ring. Polyene antibiotics show little or no antibacterial activity but are potent antifungal agents, being used extensively for treatment of systemic and non-systemic mycoses. Polyenes have also found novel applications in the treatment of certain physiological disorders.

The production of polyene antibiotics by *Streptomyces* sp. is relatively common. Almost a hundred polyene antibiotics have been described, some of which may not be unique or even pure. It would be impossible to discuss fully the biological and chemical properties of all members of the group. This review has been confined to polyenes in clinical use and those polyenes with properties that contribute to the understanding of the mechanism of action of the group as a whole.

DISCOVERY OF POLYENE ANTIBIOTICS

Brown and Hazen [1] reported in 1948 that many soil actinomycetes were antagonistic to fungi and two years later isolated nystatin, a broad spectrum

Table 3.1. DISCOVERY OF SOME IMPORTANT POLYENE ANTIBIOTICS

Antibiotic	*Year of isolation*	*Producing* Streptomyces *sp.*	*References*
Nystatin	1950	*S. noursei, S. albulus*	7
Rimocidin	1951	*S. rimosus*	8
Ascosin	1952	*S. canescus*	9
Perimycin	1952	*S. coelicolor*	10
Candicidin	1953	*S. griseus*	11
Candidin	1953	*S. viridoflavus*	12
Amphotericin B	1955	*S. nodosus*	13
Filipin complex (I–IV)	1955	*S. filipinensis*	14
Fungichromin–lagosin	1955	*S. cellulosae, S. pentaticus, S. roseoluteus*	15–17
Pimaricin	1957	*S. natalensis*	18
Hamycin	1961	*S. pimprina*	19
Candihexin	1974	*S. viridoflavus*	20

antifungal antibiotic with a polyene chromophore [2]. Several hundred reports of polyene antibiotics have been published since this date but, because of the scant evidence given by many authors, it is difficult to determine the total number of polyene antibiotics. Several estimates have been made [3–6] but when only those which have reasonable chemical documentation are counted, the present total is just under a hundred. *Table 3.1* gives details of the discovery of some of the more important polyenes.

Polyenes are almost exclusively produced by members of the Streptomycetacae, the majority by *Streptomyces* sp. but with a small minority by *Streptoverticillium* or *Chainia* spp. It is often claimed that polyene production is a characteristic of Waksman's BII (verticil- and melanin-positive) *Streptomyces* group. Hamilton-Miller [6] examined this statement and found that the higher incidence of polyene-producing strains in this group was statistically significant. However, polyenes are so often produced by *Streptomyces* [21–23], even by strains developed to produce non-polyene antibiotics, that polyene production is of little aid to taxonomy.

CHEMISTRY AND CLASSIFICATION OF POLYENES

In organic solvents, polyene antibiotics can be characterised by closely defined UV absorption maxima associated with the possession of a chromophore formed by the highly conjugated unsaturated structure of the molecule. Fully saturated

organic molecules contain only tightly bound electrons allowing them to absorb light only of very short wavelength. Unsaturation in the molecule, involving relatively loosely bound electrons, causes absorption to occur at longer wavelengths. Conjugation increases this bathochromic effect. The conjugated double bonds present in the polyene macrolide ring produces a strong chromophore, with sharp UV absorption maxima, allowing the polyenes to be divided into five subgroups, the trienes (with 3 double bonds in the chromophore), tetraenes (4 double bonds), pentaenes (5 double bonds), hexaenes (6 double bonds) and heptaenes (7 double bonds). For the 'classical' polyenes (those possessing an unmodified chromophore), a linear relationship exists between the three absorption maxima and the number of conjugated bonds in the chromophore (*Figure 3.1*).

The molar extinction coefficient for the absorption maxima of polyenes range from 2×10^4 to 8×10^4; so strong is the chromophore that absorption spectra can be determined for antibiotic concentrations from 0.5 to 1.0 μM. The polyene chromophore may produce a bathochromic shift strong enough for the antibiotic to appear coloured. The colour increases with the number of double bonds in the chromophore (*Table 3.2*).

Understanding of the UV absorption spectrum of polyenes has been increased

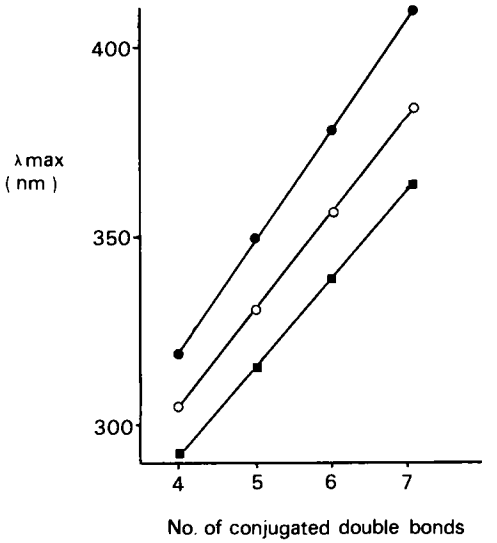


Figure 3.1. Relationship between UV absorption maxima and the number of conjugated bonds in the polyene chromophore. The wavelength of the three major absorption peaks are shown

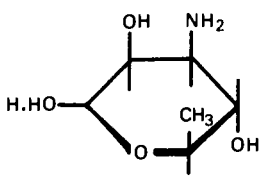
Table 3.2. UV ABSORPTION MAXIMA OF THE MAIN POLYENE SUBGROUPS

	No. of double bonds	*3 main UV absorption maxima (± 3 nm)*	*Colour*
Trienes	3	–	colourless–pale yellow
Tetraenes	4	291, 304, 318	pale yellow
Pentaenes	5	317, 331, 350	yellow
Hexaenes	6	340, 358, 380	yellow orange
Heptaenes	7	361, 382, 405	orange

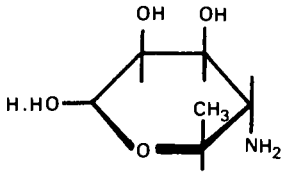
by reference to the spectrum of model hydrocarbons of known structure [4]. The recorded spectra of tetraenes, pentaenes and hexaenes suggest that the double bonds in the chromophore are all in the *trans* configuration [6]. This may also be true for certain heptaenes, e.g. candicidin and amphotericin B, but purification of the heptaene candicidin has been shown to cause a small amount of *trans* to *cis* isomerism.

UV spectroscopy also allows identification of subdivisions within the five polyene groups. Methylation adjacent to the polyene chromophore results in a bathochromic shift of the absorption maxima of about 6 nm. The presence of a ketone group adjacent to the chromophore radically alters the absorption spectrum, e.g. the carbonyl pentaene flavomycoin possesses only two absorption peaks, while the carbonyl hexaene dermostatin possesses a single peak [24–26].

A second aid to the chemical classification of polyenes is the presence in the antibiotic of either a hexosamine sugar or an aromatic moiety. The presence of sugars is a characteristic of macrolide antibiotics. Polyene hexosamines are similar to the sugars present in the non-polyene macrolides [27]. The structure of polyene hexosamines has been determined and with one exception, the carbohydrate is mycosamine [28–30], the structure of which has been unequivocally established by chemical synthesis [31] as 3-amino-3,6-dideoxy-D-mannopyranose (1). The sugar perosamine, 4-amino-4,6-dideoxy-D-mannose (2), an isomer of mycosamine, has been isolated from perimycin [31,32].

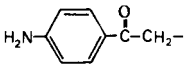


(1)

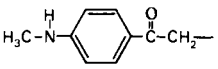


(2)

Certain members of the heptaene subgroup, e.g. candicidin, ascosin and hamycin, contain an aromatic amine sidechain *p*-aminoacetophenone (3), which is alkali-labile [33–36]. In other heptaenes, e.g. candimycin and perimycin, the aromatic sidechain is *N*-methylated (4) [34].



(3)



(4)

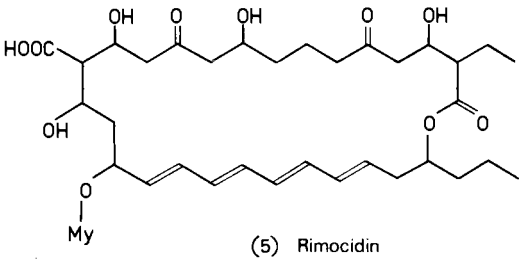
The presence of mycosamine or perosamine confers a basic character on the polyene molecule. Many of the polyene antibiotics, e.g. nystatin, amphotericin B and candicidin are amphoteric, possessing an equal number of basic (hexosamine or aromatic sidechain) and acidic (carboxyl) groups. Other polyenes, e.g. filipin and fungichromin, possess no ionizable groups at all, i.e. they are non-polar.

TRIENES

Five macrolide antibiotics with a triene chromophore have been reported, MM88, mycotrienin, proticin, resistaphylin and trienine [37–42]. Although Hamilton-Miller [6] included the trienes with the polyenes, this group exhibits many atypical properties. Resistaphylin and proticin are cytotoxic, show no antifungal activity, and yet are strongly bacteriostatic. Mycotrienin and MM88 do not possess a carbohydrate moiety. Proticin, isolated from *Baccilus licheniformis* var. *mesentericus* is unique in containing phosphorus and being synthesised outside the *Streptomyces* group [41,42]. Trienes show a greater affinity towards the non-polyene macrolide antibiotics, e.g. erythromycin, than to the other polyene groups. This group will not be discussed further.

TETRAENES

This is a larger group of antibiotics; of over twenty described tetraenes, structures have been proposed for only six. The majority of the group, including tetrin A and B, and rimocidin (5) contain a macrolide ring of 25 to 27 carbon

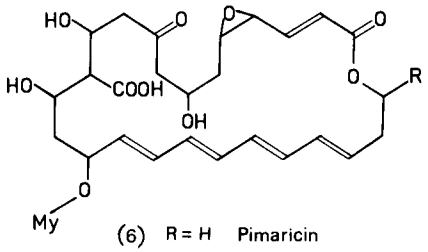


(5) Rimocidin
My = mycosamine

Table 3.3. CHEMICAL COMPOSITION OF TETRAENES

	Antibiotic	*Chemical composition (mol. wt.)*	*No. of carbon atoms in macrolide ring*	*Reference*
'Classical' tetraenes	Tetrin A	$C_{34}H_{51}NO_{13}$ (681)	25	43
	Tetrin B	$C_{34}H_{51}NO_{14}$ (697)	25	44
	Rimocidin	$C_{38}H_{63}NO_{13}$ (742)	27	45, 46
Epoxide tetraenes	Pimaricin	$C_{33}H_{47}NO_{14}$ (666)	25	47
	Etruscomycin	$C_{36}H_{53}NO_{13}$ (708)	25	48, 49
'Degenerate heptaenes'	Nystatin	$C_{47}H_{75}NO_{17}$ (926)	37	50

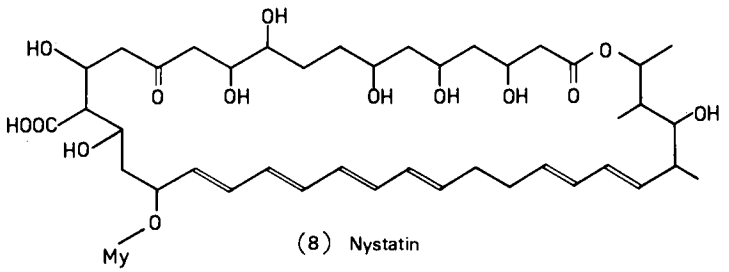
atoms (*Table 3.3*). Pimaricin (6) and etruscomycin (luscensomycin) (7) can be separated from the 'classical' tetraenes by the possession of an oxirane ring. This second ring system, uncommon in macrolide antibiotics, makes this group, the epoxide tetraenes, unique amongst polyenes.



(6) R = H Pimaricin

(7) R = $(CH_2)_2$ Me Etruscomycin

Nystatin (8) possess a much larger macrolide ring (*Table 3.3*), with little structural affinity to rimocidin (5), pimaricin (6) or etruscomycin (7) but resembles the structure of the heptaenes amphotericin B (11) and candidin (12). Nystatin, classed as a tetraene because of the UV absorption of the four con-



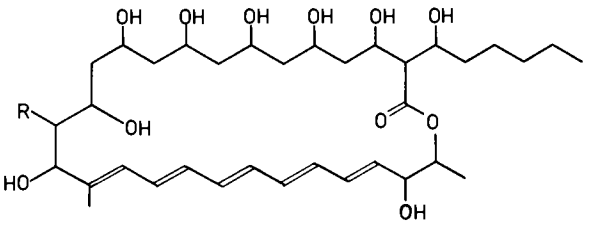
(8) Nystatin

jugated double bonds in the molecule, also contains a further two conjugated bonds separated from the four by a methylene bridge. In all its properties, except its UV absorption spectrum, nystatin behaves as though it were a heptaene, modified by the saturation of one double bond.

Counter current distribution studies have shown that nystatin consists of two components designated nystatin A_1 and A_2 [51]. Nystatin A_1 is considerably more stable than A_2 and the ratio of A_1 to A_2 decides the stability of the sample. High speed liquid chromatography [52] has also resolved nystatin into two tetraenes and also revealed a heptane component.

PENTAENES

This is the largest group with over 30 described members. It contains, in addition to the classical unsubstituted polyenes, e.g. fungichromatin, the lactone conjugated carbonyl-pentaenes recognisable by a modified UV absorption spectrum, e.g. flavomycoin and the methyl pentaenes, recognisable by a characteristic infra-red absorption spectrum [53]. The latter group includes filipin (9) and fungichromin–lagosin (10). Filipin has been separated by column chromatog-



(9) R =H Filipin
(10) R =OH Fungichromin

raphy into four main components designated I, II, III and IV [54]. Further, filipin I comprised a mixture of an additional five components which were isolated as a crystalline mixture. After separation of the filipin complex, analysis of the remaining material indicated the presence of seven more filipin-like compounds. The antifungal activity of the filipin complex was the consequence of the presence of filipin II and III as major fractions. It was found that the relative proportions of the components were: filipin 4%, filipin II 25%, filipin III 53% and filipin IV 18%. The four components contain a tetraene chromophore and have been characterised by mass spectroscopy [55]. Investigations into the mechanism of action of filipin made before this observation (in 1968) refer to an unspecified filipin complex.

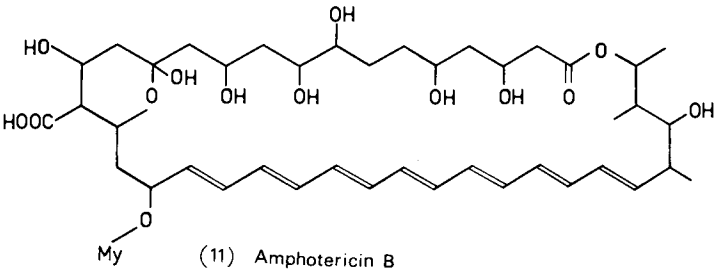
HEXAENES

This small group of antibiotics has received relatively little attention. Dermostatin has been shown to possess a conjugated hexaene-ketone chromophore and does not contain carbohydrate [26,56]. The recent discovery of candihexin [20], which has certain novel properties, has increased interest in this group. Candihexin has been resolved into eight components [57], designated A–F, of which A, B, E and F constitute 90% of the total. Candihexins A and B were found to have the highest antifungal activity [57].

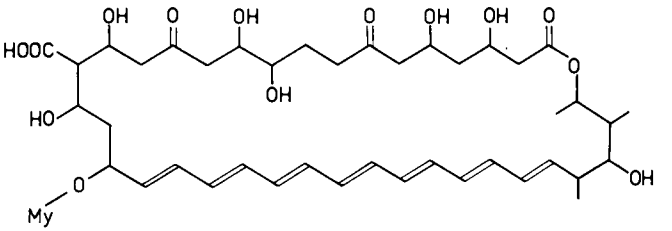
HEPTAENES

This group contains over 30 described members, including two of clinical importance, amphotericin B and candicidin. Heptaenes can be divided into three subgroups on the basis of the presence or absence of an aromatic sidechain.

Some heptaenes, e.g. amphotericin B (11) and candidin (12), contain no



(11) Amphotericin B



(12) Candidin

aromatic moiety [58,59]. The antibiotic originally described as candidin [60] has since been recognised as a mixture of three components [59]; the name candidin was retained for the principal component and the others designated candididin and candidoin. Reference in this review is to this later, more pure, candidin.

The heptaenes of the candicidin group [61], candicidin, hamycin and ascosin, contain an aromatic sidechain (3) derived from *p*-aminoacetophenone [9,34,35].

The third group, which includes perimycin and candimycin, also possesses an aromatic sidechain but in this case it is *N*-methylated as in formula (4) [63]. Perimycin also contains a unique amino sugar, perosamine (2) [31].

Table 3.4 gives a classification scheme for the polyene antibiotics based on their physico-chemical properties. Although such a classification is useful, it will

Table 3.4. CLASSIFICATION OF POLYENE ANTIBIOTICS ON THE BASIS OF PHYSICO-CHEMICAL PROPERTIES

	λ_{max} (± 2 nm)	*Antibiotic*	*Sugar moiety*	*References*
Tetraenes				
'Classical' tetraenes	291, 304, 318	Rimocidin	M	8,45,46,62
Epoxide tetraenes	290, 303, 318	Pimaricin	M	47,63–65
		Etruscomycin	M	48,49,66
'Degenerate' heptaene	292, 304, 318	Nystatin	M	50,67
Pentaenes				
'Classical' pentaenes	317, 331, 350	Eurocidin	M	68
		Fungichromatin	?	15
Methyl pentaenes	323, 340, 357	Filipin complex (I–IV)	None	14,54,55,69–73
		Fungichromin–lagosin	None	16,17,73
Carbonyl pentaenes	364 (single broad peak)	Flavomycoin	?	25
Hexaenes				
'Classical' hexaenes	340, 358, 380	Endomycin B	?	74
		Candihexin	?	57
Carbonyl hexaenes	385 (single broad peak)	Dermostatin	None	26,56
Heptaenes				
'Classical' heptaenes	363, 382, 406	Amphotericin B	M	58,75
		Candidin	M	59
Aromatic heptaenes	360, 380, 402	Ascosin		9
(*p*-amino-acetophenone)		Candicidin	M	34,61
		Hamycin		35
(*N*-Methyl-*p*-amino-acetophenone)	361, 383, 406	Perimycin	P	32,76

M, mycosamine; P, perosamine.

be shown that biological properties of the group do not always reflect this classification.

SOLUBILITY PROPERTIES

The structure of polyene antibiotics reveals a feature of importance to their physical properties. On one side of the macrolide ring is a series of hydroxyl groups, while on the opposite side of the ring is located the unsaturated polyene chromophore. The chromophore is thoroughly hydrophobic, while the remainder of the molecule is hydrophilic. Solubility decreases from the tetraenes to the heptaenes, with the increasing hydrophobic character of the chromophore. Polyenes have limited solubility in water or non-polar organic solvents, e.g. alcohols, ketones, esters and ethers, but are soluble in polar organic solvents, e.g. dimethyl sulphoxide, dimethyl formamide or aqueous alcohol mixtures. Solutions of polyenes in polar organic solvents may then be diluted with water to give stable solutions, when the final antibiotic concentration is less than 50 μg/ml. Such diluted solutions are not dialysable and are believed not to be true solutions but to exist as micelles in an aqueous environment [77,78].

STABILITY

The statement that polyene antibiotics exhibit poor stability to heat, UV radiation and extremes of pH is often made [34,79–81]. When pure dry antibiotic is stored in the dark at low temperature, potency will not be lost, even over long periods [82,83]. Stability to light and temperature decreases as the size of the chromophore increases.

Some polyenes, e.g. pimaricin, are more thermostable than others [18]. Thermal inactivation of nystatin was associated with loss of the polyene chromophore but was reduced by addition of antioxidant [84]. Hamilton-Miller [6] cites several reports showing that light over a large wavelength range causes photo-deterioration of polyenes, but that the most damaging radiations were between 380 and 410 nm, corresponding to the absorption maxima of the polyene chromophore. Polyenes undergo oxidative degradation in the presence of air and light to give epoxides, which on extended oxidation produce higher oxidation products and ultimately polymeric material [72].

The addition of antioxidants considerably prolonged the active life of nystatin, filipin and lagosin. It has been suggested that UV photolysis induced *trans* to *cis* isomerism [85]. Photo-oxidation of aqueous pimaricin solutions was stimulated by low concentrations of flavèin derivatives [86]. Inactivation of nystatin in dimethyl formamide has been shown to be due to peroxides present in the solvent [87].

The instability of polyenes should not be overstated; the dry product is relatively stable and it has been shown that aqueous solutions of polyenes are stable at least for 24 h even when exposed to light, air and room temperatures [88–90]. When dissolved in aqueous systems, the antibiotic consists of a micellar dispersion held together by hydrophobic forces. This may confer a degree of protection in that the sensitive region of the antibiotic exists in a localised hydrophobic environment shielded from physical and chemical damage. When stored under identical conditions, dry amphotericin B and amphotericin B methyl ester exhibited similar stability. In aqueous solution, amphotericin B was more stable than its methyl ester. Spectrophotometric studies [91] have shown that in aqueous solutions amphotericin methyl ester was more dispersed than the parent compound and it appeared that increased water solubility was accompanied by increased vulnerability to damage [92].

BIOSYNTHESIS OF POLYENE ANTIBIOTICS

The mechanisms of polyene antibiotic biosynthesis have received little attention. Although the polyketide route, the major pathway in the biosynthesis of fungal secondary metabolites, is uncommon in actinomycetales [93], a variation of this pathway, the polyproprionate route (involving linear condensation of propionic and methyl malonic acids, in the place of acetic and malonic acids), is commonly found [94]. The aglycone ring of polyenes has been shown to be derived from acetate and propionate units, i.e. it involves both the polyketide and polypropionate route [95–97]. Nystatin and amphotericin B are assembled from three propionate and sixteen acetate units [96,98] while etrusmycin is assembled from two propionate and twelve acetate units [97], with a single propionate unit providing the chain initiating unit. The methyl sidechains of nystatin and candicidin have been shown to be derived from propionate [95,96].

A feature of actinomycelates biosynthesis is the incorporation of the intact carbon skeleton of glucose into secondary metabolites. It appears that mycosamine is derived from D-glucose and is incorporated into the polyene molecule unchanged [95–97,99].

The shikimate pathway is the major route to aromatic compounds in actinomycetales. Biosynthesis of the aromatic side chains of candicidin [95] and perimycin [100] has been shown to arise directly from glucose via shikimate and *p*-aminobenzoate.

MECHANISM OF ACTION OF POLYENE ANTIBIOTICS

In 1958 two important observations were made which proved fundamental to the understanding of the mechanism of action of polyene antibiotics. It was

demonstrated [101] that nystatin damaged the membranes of yeast in some subtle way, which allowed the leakage of low molecular weight material from the cell, while retaining larger molecules. It was also shown that addition of sterols to growth medium was able to protect fungi against polyene antibiotics [102]. Later work has demonstrated that polyene antibiotics bring about their lethal effect by combination with cell membrane sterol, altering the selective permeability properties of the membrane, allowing the leakage of essential metabolites, and destroying the ability of the membrane to control nutrient uptake.

INTERACTION BETWEEN POLYENE ANTIBIOTICS AND STEROLS

Several excellent reviews have described in detail the role of sterols in the mechanism of action of polyenes [103–105]. Only a summary of the evidence will be given.

EVIDENCE FOR POLYENE–STEROL INTERACTION

Only sensitive organisms contain sterol

Polyene antibiotics were absorbed by, and were lethal to, eukaryotic organisms including yeasts [106–108], mycellating fungi [109,110] cellular slime moulds [111], protozoa [112,113], green algae [114], planarian worms [115], and snails [116]. Polyenes also brought about haemolysis of mammalian erythrocytes [117–119] and were toxic to mammalian cells in tissue culture [120,121]. Prokaryotic organisms, e.g. bacteria [77,122–124] and blue-green algae [114], neither absorbed or were affected by polyenes. Sterols, present in the membranes of polyene-sensitive eukaryotes [125–127], were absent from insensitive prokaryotes [128–131]. The possibility that bacterial cells possessed the same component, but that accessibility to binding sites was denied by the bacterial cell wall or the bacterium in some way destroyed the antibiotic, was eliminated when it was shown that bacterial protoplasts, like the intact cells from which they are derived, did not leak cytoplasmic components or absorb polyenes; unchanged antibiotic was recovered from the medium [124,132,133].

Bacteria belonging to the Mycoplasmatales, e.g. *Acholeoplasma laidlawii* do not require sterols for growth but can incorporate them into the plasma membrane when grown in sterol-containing medium. *A. laidlawii* cells were only sensitive to polyene antibiotics (filipin and amphotericin B) when the organism contained sterol [134,135]. Similarly polyenes have been shown to possess activity against a protoplast L-form of *Escherichia coli* W1655F$^+$ but not the

normal rod form [136]. Both the normal rod form and the protoplast were found to contain sterols, and it was suggested that in this case the bacterial wall masks the sterol target site in the normal rod form. With these two exceptions, bacteria tolerate extremely high concentrations of polyene antibiotic.

In fungi, increases in sterol content also increases polyene sensitivity; when the yeast *Schizosaccharomyces japonicus* was grown aerobically it contained ergosterol and was sensitive to polyenes, but when grown anaerobically this yeast lacked sterol and became polyene-insensitive [137]. When the sterol level of *Candida albicans* was increased, this was accompanied by increased antibiotic sensitivity [138]. In yeasts, lowered sterol levels resulted in greater resistance to polyene antibiotics [138,139].

Antifungal action of polyenes is inhibited by added sterols

Addition of sterols, particularly those with a 3β-hydroxyl group, a planar sterol nucleus and a long C_{17} sidechain, to growth medium inhibited the antifungal action of polyene antibiotics [102,140–143]. Sterol addition prevented polyene uptake by yeasts [143], suggesting that added sterol complexes with the antibiotic, thereby lowering the effective antibiotic concentration, although such a simple interaction has been disputed [142,144].

Polyene–sterol interaction has been demonstrated in vitro

There is limited spectroscopic evidence for the direct interaction of sterols and polyenes [141,143,145–147]. The characteristic UV absorption spectrum of individual polyenes in aqueous and organic solvents was modified by addition of free sterols. Sterol addition significantly lowered the extinction coefficient of the polyene UV absorption maxima [146–151]. Sterols most effective in preventing the growth inhibition of fungi by polyenes [102,140–143] were found to produce the greatest effect on the absorption spectrum [143]. It has been shown that filipin, nystatin, etruscomycin, amphotericin B and pimaricin all interact with cholesterol. The order of effectiveness of cholesterol addition in reducing the UV absorption maxima was filipin > amphotericin B > etruscomycin > nystatin = pimaricin [146,152–155]. Sterol esters were found not to interact with polyenes to the same extent. Sterols with an intact cholestane ring, a 3β-hydroxyl group, an intact sidechain at C_{17}, and a Δ-22 double bond produced optimum interaction [146]. Similar changes to the UV absorption spectra of filipin, etruscomycin and amphotericin B were observed on addition of antibiotics to liposomal, erythrocyte or *A. laidlawii* membrane-bound cholesterol. No spectral change was observed with sterol-free membranes. However, as polyenes do not exist as true solutions but as micelles [77,78,143,146],

spectrophotometric data were not thought conclusive but only indicative of an interaction.

Spectrofluorometry gives more direct evidence for sterol–polyene interaction [150,151]. This technique, by measuring partial quantum efficiency, eliminated the problems inherent in measuring changes in light absorption of compounds which are sparingly soluble in water. Amphoteric polyenes, e.g. nystatin, amphotericin B, etruscomycin and pimaricin show little intrinsic fluorescence but on addition of β-hydroxylated sterols, fluorescence increased [146,147,156–158]. In contrast, the fluorescence of non-ionized pentaenes, e.g. filipin and lagosin, which was relatively high, decreased in the presence of sterols [149]. Spectrofluorometry has given results in general agreement with polyene-induced changes in UV absorption spectrum.

Examination of the spectral changes resulting from addition of sterol to filipin solutions suggests that filipin interacts with sterol to produce a filipin–sterol complex, in which the cholesterol molecules are in close association with the conjugated bond of the filipin chromophore [146,149]. The filipin–sterol complex can be easily broken by treatment with detergents or water-miscible organic solvents, e.g. methanol or dioxan. It has been demonstrated using fluorescence polarization studies that, when polyene antibiotics combined with sterols, the rigidity of the polyene antibiotic increased [157,159].

Membranes extracted with organic solvents to remove sterol fail to bind polyenes

The bulk of nystatin absorbed by *Neurospora crassa* was found to be located in the microsomal fraction of cell-free homogenates [124,132,160]. Extraction with organic solvents abolished the affinity of this membrane fraction for the antibiotic. The ability to bind nystatin was restored by pre-incubation of extracted particles with ergosterol.

Sterol-complexing agents interfere with polyene activity

Polyene absorption by fungal membranes can be inhibited by agents with a high affinity for sterols, e.g. digitonin [161–164]. Digitonin also releases bound polyenes from membranes.

Sterols are not the only conceivable membrane constituent which have been reported to antagonise polyenes or inhibit polyene binding. Similar effects have also been reported for oleic acid, linoleic acid, vitamin A, and lecithins [162, 165–169]. Such compounds may compete with sterol in the cell membrane for available polyene molecules or more likely bind to the cell surface, and there they either interact with the polyene antibiotic or mask sterol molecules in the membrane, thereby reducing the number of binding sites. It has been shown that

filipin can interact with cetyl alcohol and to a lesser extent with cerebrosides [170]. However, sterols remain the only compounds whose phylogenic distribution, localisation in cell membranes and physico-chemical properties account for the observed biological properties of polyenes.

STOICHIOMETRY OF THE POLYENE–STEROL INTERACTION

The stoichiometry of the polyene antibiotic–cholesterol interaction has been measured in various systems with different techniques. The filipin–cholesterol interaction has been the subject of extensive study and the results suggest this interaction is equimolar. The data on other polyenes is not so definite, but suggests that only a small number of sterol molecules interact with one polyene antibiotic molecule.

Fluorometric and spectrophotometric studies of filipin–cholesterol interaction showed that the stoichiometry of the interaction was 1 : 1 [150] or 1 : 1.5 [146,147]. UV spectrophotometry changes have been used to monitor the stoichiometry of the interaction between filipin and free or liposome-bound cholesterol. Analysis of aqueous dispersions suggested that the stoichiometry was 1 : 1 [171]. Lecithin, dicetyl phosphate–cholesterol liposomes only produced maximal spectral changes of filipin when the sterol : polyene ratio was 1 : 1 [172]. Filipin released trapped ion markers from sterol–phospholipid liposomes. The rate of release was dependent upon cholesterol content of the liposome membrane (maximum at sterol : phospholipid ratio of 1 : 1) and upon the molar filipin : sterol ratio (maximum at filipin : sterol ratio of 1 : 1).

Estimates of the stoichiometry of polyene–sterol complex can be made from differential scanning calorimetry (DSC) of lecithin–sterol dispersions [146]. When the values obtained by DSC [146] were corrected for the amount of cholesterol which would be unavailable for polyene binding [154], it was shown that the number of cholesterol molecules that could be complexed or associated with filipin, etruscomycin, pimaricin, nystatin and amphotericin B were 1.2, 0.6, 1.7, 1.2 and 3.9, respectively. Permeability studies using cholesterol containing *A. laidlawii* cells [173] indicated a similar stoichiometry (filipin 0.7, etruscomycin, 0.3, nystatin, 1.6 and amphotericin B, 3.3). Measurement of the binding of various polyene antibiotics to cholesterol-containing *A. laidlawii* membranes also gave an estimate of the number of cholesterol molecules that combine with filipin, etruscomycin, pimaricin and amphotericin B. The values obtained were 1.2, 1.6, 1.5 and 0.7, respectively [153].

Schaffner [145] showed that addition of cholesterol or other 3β-OH, Δ-5 sterols to aqueous ethanolic solutions of polyene antibiotics produced changes in the UV absorption spectrum suggestive of molecular interaction. In aqueous

ethanolic solution sterol addition solubilized amphotericin B and this interaction increased until the molar ratio of cholesterol : amphotericin B approaches 2 : 1.

POLYENE ANTIBIOTICS AS MEMBRANE-ACTIVE AGENTS

The functions of the cell membrane are varied. It forms an expandable and protective barrier to the protoplast, acts as an organelle controlling the entry and exit of solute molecules, and provides a site for synthesis at which cell wall and extra-mural layers are assembled. These manifold functions of the membrane make it vulnerable to a variety of agents and the disturbance of membrane function is often lethal. Several antimicrobial agents produce their effect by acting upon the cell membrane, including the surfactants, e.g. cetrimide, peptide antibiotics, e.g. polymixins, gramicidin, tyrocidin, valinomycin and the polyene antibiotics [174]. Membrane-active agents generally alter the selective permeability of the cell membrane leading to loss of cytoplasmic components.

The observation that polyenes alter the selective permeability properties of eukaryote membranes [101] has resulted in extensive studies on the interaction between polyenes and the sterol component of membranes. Valuable information on the mechanism of polyene–membrane interaction has been obtained not only from intact cell membranes and membranes derived from cells but also from artificial membrane systems of known lipid composition.

INTERACTION WITH LIPOSOMES

Liposome is the general name for specially prepared lipid dispersions in water (alternatively known as smetic mesophases, 'Bangosomes', liquid crystals, or liquid sphaerules), prepared by allowing lecithin to swell in water or by dissolving lecithin, dicetyl phosphate (and if required, sterols) in organic solvents, evaporating, suspending in aqueous solvents and homogenising the mixture. Liposomes are usually spherical (0.5–50 μm diameter) and consist of several bimolecular layers. The permeability properties of liposomes can be studied by trapping ions, nonelectrolytes or radioactive markers within the liposome and following their subsequent release or by observing their rates of swelling in hypertonic solutions [175]. Liposomes show low permeability to ions but high permeability to small nonelectrolytes [176]. The lipid composition of liposomes can be varied under controlled conditions and the influence of variations on the liposome permeability investigated. Sterol-containing liposomes showed reduced ionic permeabilities [152,177–180]. Lecithin liposomes were insensitive to polyenes, while sterol-containing liposomes have been shown to leak ions and sugars soon after polyene treatment.

By the use of a sensitive glucose assay, it was demonstrated that the presence of cholesterol in liposomes increased the leakage of a glucose marker induced by low concentrations ($10^{-6}-10^{-5}$ M) of filipin [181]. In earlier papers Weissmann and Sessa [182,183] also followed the release of trapped glucose, chromate and phosphate markers from liposomes prepared with and without cholesterol. Treatment with higher concentrations ($10^{-4}-10^{-3}$ M) of nystatin, amphotericin B, etruscomycin and filipin enhanced the rate of marker release in sterol-containing liposomes. These workers also reported an interaction between filipin and pure lecithin liposomes without sterols [182]. The heterogenous nature of the filipin complex [54] was not realised at this time and it was later shown that the small filipin II component was responsible for the non-sterol-specific binding [184]. It is not readily apparent why filipin II at the higher concentrations used by Weissmann and Sessa interacts with pure lecithin liposomes but when used by others [181] at lower concentrations produce no effect. It may be that filipin II has a greater ability to form true solutions than other components of the filipin complex and when the filipin II concentration is high it has a non-specific detergent effect on liposomes [132]. Attempts to demonstrate interaction between pimaricin or etruscomycin and cholesterol-containing liposomes have not been successful [181,182]. It has been demonstrated that incorporation of cholesterol into egg lecithin liposomes enhanced the sensitivity of the liposome to amphotericin B but that the polyene sensitivity of dipalmitoyl lecithin liposomes decreased after sterol incorporation. It has been suggested that the liposome membrane exists in an ordered state for polyene–sterol interaction and cholesterol makes egg lecithin liposomes more ordered while decreasing the order of dipalmatoyl lecithin liposomes [185].

Sterol-supplemented liposomes were able to bind and alter the UV absorption spectra of filipin, etruscomycin and amphotericin B but not those of nystatin or pimaricin [146]. The presence of a 3β-hydroxyl group in the sterol incorporated into the liposome appeared to be a prerequisite for interaction with polyene antibiotics. Sterol-containing liposomes have been shown to enhance the fluorescence intensity of filipin and etruscomycin [157,186].

The nature of the interaction between polyene antibiotics and membrane sterols has also been investigated using UV, fluorescence and circular dichroism spectral studies [155,159,187,188]. The rate and degree of filipin III binding to cholesterol-containing liposomes depended upon the absolute sterol concentration and the mole percent in the bilayer. Binding constants for filipin III and liposomes were also estimated.

Liposomes, prepared by extracting naturally occurring membranes of *A. laidlawii* grown with and without sterol, possessed permeability properties comparable with those of parent cells [152]. To reduce the permeability of the liposome, the sterol must possess a planar sterol ring and 3β-hydroxyl group [179,

189]. The polyene sterol interaction did not merely nullify the effect of sterol addition. Polyenes appeared to combine with liposome sterols and this combination destroyed the permeability properties of the liposome.

The permeability properties of liposomes are related to the physical state of the constituent hydrocarbon chains. It is thought that sterol incorporation decreases overall liposome permeability to nonelectrolytes by affecting the dehydration of the molecule as it enters the membrane, rather than by slowing subsequent solute diffusion. Studies into the interaction between low concentrations of amphotericin B, nystatin and filipin with cholesterol-containing single bilayer lipid vesicles have demonstrated that these antibiotics caused extensive changes in liposome structure. Nystatin and amphotericin B induce rapid loss of vesicle contents, consistent with the formation of small pores in the membrane [190]. In contrast, filipin was found to destroy vesicle structure so rapidly that its action was likened to the action of a simple detergent [191]. Although the presence of cholesterol was required for polyene interaction, NMR studies indicated that the polyene–sterol interaction did not reverse the cholesterol restriction on phospholipid mobility [191]. This conflicts with calorimetric results using liposomes, which have shown that polyenes reversed the depression of the gel to liquid-crystalline transitional enthalpies induced by cholesterol [153].

INTERACTION WITH MONOLAYER AND BILAYER SYSTEMS

Membrane-active antimicrobial agents can react with monomolecular lipid films (or monolayers) orientated at an air–water interface. When such an agent is introduced beneath the monolayer, the orientation of the lipid molecules at the interface alters, producing measurable changes in surface pressure.

Low concentrations (10^{-8} M) of filipin or nystatin did not react with monolayers prepared from bacterial protoplast membranes or pure lecithin monolayers but did increase the surface pressure of monolayers which contained ergosterol or cholesterol [192]. Later work [193] demonstrated that a wider range of nystatin, filipin, pimaricin or amphotericin B concentrations showed no interaction with pure phospholipid monolayers but markedly increased the surface pressure of monolayers containing cholesterol and to a lesser extent those containing ergosterol. Sterol incorporation has a condensing effect on lecithin monolayers, i.e. facilitates close packing of the constituent lipids [194–196]. This close packing was reversed by polyene–sterol interaction. Esterification of added sterol or addition of 5 M urea lowered the pressure change resulting from filipin addition [193], suggesting that the polyene–sterol complex is stabilised by hydrogen bonding. Hydrogenation, irradiation (which destroy the polyene chromophore) or saponification (which breaks the macro-

lide ring) of filipin reduced the potency of the antibiotic [197] and these derivatives produced smaller increases in the surface pressure of cholesterol monolayers than the parent antibiotic [193].

Monolayer studies provide additional information on the principles behind the selective toxicity of polyenes towards organisms with sterol-containing membranes but do not give an indication of the nature or structure of polyene membrane damage. The surface pressure changes elicited in monolayers may reflect accumulation of antibiotic molecules beneath the monolayer or penetration of the monolayer by the antibiotic, either of which could change the orientation of sterol molecules [132].

Monolayers are only representative of half a membrane. The structure and permeability properties of stable bilayer membranes are more characteristic of living cell membranes than simple monolayers and may provide a model for polyene–membrane sterol interaction. Bilayer membranes are very thin lipid films formed across a small orifice in a septum separating two aqueous phases and are so called because they are approximately twice as thick as monolayers [198]. Because such films fail to reflect light and so appear black when viewed against dark backgrounds they have been termed secondary black membranes. The permeability properties of bilayer membranes are studied by measuring the ability of the film to prevent an excess of an ionic solution, e.g. 0.1 M sodium chloride, placed on one side of the membrane passing through the bilayer. As the film separates the two chambers, it also permits the monitoring of capacitance or potential differences existing across the bilayer.

The stability of bilayers in the presence of polyene antibiotics has been investigated [199]. Pure lecithin bilayers were found to be stable in the presence of 40 μM filipin or nystatin; in contrast, bilayers consisting of equal parts cholesterol and lecithin were disrupted rapidly by filipin but considerably more slowly by nystatin. Bilayers consisting exclusively of cholesterol or bilayers with phospholipid : sterol ratios of 10 : 1 were stable in the presence of filipin. When added to a sterol-containing lecithin bilayer, filipin removed the sterol from the membrane by non-polar association, changing the fluidity of the lipid chains in the bilayer, and ultimately caused membrane breakdown [200].

Several investigations have been made into the electrical consequences of adding various polyenes to bilayer preparations [201–205]. Nystatin and amphotericin B increased the permeability of bilayers to various ions, water and nonelectrolytes [201–208]. It is suggested that polyenes create areas of disturbance in the bilayer through which small molecules could migrate. Addition of polyene antibiotics mediated changes in the electrical properties of the bilayer by causing an asymmetric distribution of the charged ion. Low polyene concentrations (10^{-7}–10^{-5} M) were found to lower the d.c. resistance of the bilayer by up to a million fold. The extent of resistance change depended upon tempera-

ture and antibiotic concentration. Polyenes were found to have no effect on the d.c. resistance of bilayers which did not contain sterol concentrations above a critical level. Nystatin and amphotericin B appeared to have a selective action on sterol–lecithin bilayers, in that the permeability anions (chloride or acetate) increased much more than cation (potassium or sodium) permeability, i.e. most of the current was carried across the antibiotic-modified membrane by anions [204,205,209].

Filipin complex, filipin II, filipin III, nystatin, etruscomycin and pimaricin solutions (10^{-5} M) disrupted bimolecular films which contained equimolar concentrations of cholesterol and lecithin but had no effect on pure lecithin bilayers. Filipin, which had been hydrogenated or exposed to UV radiation, failed to disrupt bilayers prepared with or without sterols [210]. Similarly amphotericin B increased the potassium permeability of bilayer membranes to a greater extent than hydrogenated, saponified or acetylated amphotericin B [211]. The presence of calcium ions was found to reduce the d.c. resistance of filipin-, nystatin- or primaricin-treated lecithin–cholesterol bilayers (in 0.1 M NaCl) by a factor of 10^4–10^5. Other cations or anions did not reduce the d.c. resistance of the bilayer in the presence of polyenes. Transference number measurements (defined as the ratio of the total current carried by a single ionic species) suggested that under these conditions polyene-treated bilayers become cation-selective [210]. Transference number measurements are based on the assumption that electric charge is transferred by the ions and the driving force is a difference in electrical potential or salt concentration between the two sides.

Addition of sterol to bilayer membranes dramatically decreased both ionic and non-ionic permeability [212] but interaction of polyenes with membrane sterol did more than simply reverse the effect of sterol incorporation. Polyene addition produced specific permeability alterations [201–205] consistent with the creation of ion-selective channels or pores within the bilayer.

It is interesting to note than the structural requirement for the maximal interaction between sterols and lecithin monolayers [196] and for sterol-mediated reduction of liposome permeability [179] are a 3β-hydroxyl group, a planar sterol nucleus and an intact C_{17} sidechain, which is identical with the requirement for maximal polyene sterol interaction [101,146].

DIFFERENTIAL SCANNING CALORIMETRY (DSC) STUDIES

Preparations of synthesised lecithins with defined fatty acid composition exhibit a sharp transition over a narrow temperature range characteristic of a conversion from an ordered crystalline to a more random crystalline state (*Figure 3.2*). Such transitions can be followed using DSC. The exact temperature of this transition depended upon the fatty acid composition [213]. Incorporation of sterol into

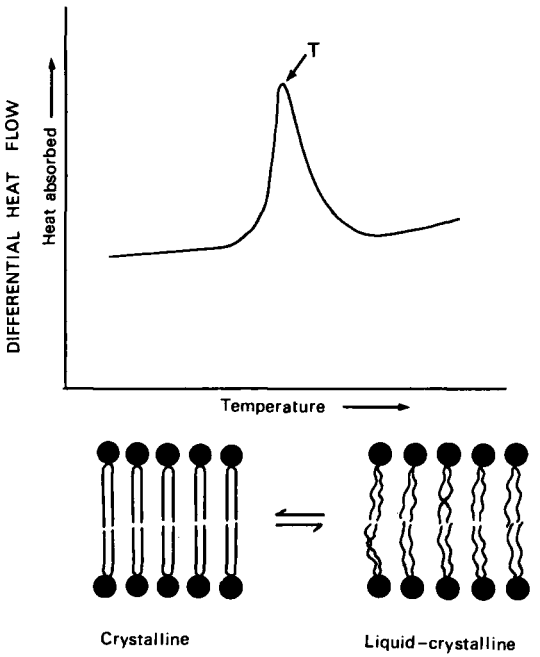


Figure 3.2. Idealized calorimetric scan of lipids indicating endothermic transitions from crystalline state at lower temperatures to liquid-crystalline phase above the transition temperature (T)

the synthetic lecithin led to a fluidization of the lecithin hydrocarbon chains and abolishes this phase transition [214]. Addition of filipin to lecithin–cholesterol dispersions resulted in a re-appearance of the phase-transition characteristics of pure lecithin dispersions [146]. Addition of filipin to pure lecithin dispersions did not change the phase transitions. The formation of the filipin–sterol complex in the lecithin dispersion destroyed the fluidity in the lecithin hydrocarbon chains imparted by sterol addition, enabling the dispersion to undergo, once more, a phase transition [147]. Later work indicated that etruscomycin, pimaricin, nystatin and amphotericin B were also capable of causing the re-appearance of phase transitions in lecithin–cholesterol dispersions [146]. DSC has also been used to examine the effects of polyene antibiotics on the thermodynamics of the phase transitions in sterol-containing membranes of *A. laidlawii* [153].

Other studies [215] have suggested that the lecithin in the dispersion interacts with filipin thereby providing a matrix for interaction of the antibiotic with the sterol. There was no evidence (from infra-red spectroscopy) for hydrogen

bonding between filipin and sterol or filipin and lecithin, although there was some indication from thermal measurements that filipin interacts with the polar group of the phospholipid [200].

Whether phase transition changes are directly responsible for the permeability changes observed in liposomes and bilayers is debatable but results obtained by DSC are indicative that polyene addition to sterol-containing liposomes, bilayers or membranes markedly alters the molecular structure of the membrane by reducing the phospholipid–sterol interaction. Such alterations may be the basis for the observed polyene-mediated permeability changes to ions and small non-electrolytes [101,103–106,201–212,216].

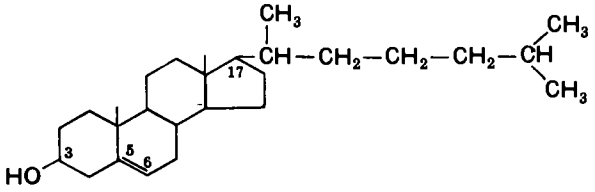
ROLE OF STEROLS IN EUKARYOTE MEMBRANES

Eukaryotic membranes are believed to consist of three layers, an outer and inner layer of hydrophilic material of which protein composes a major part and a central lipid portion consisting of two regions of lipoid material lying with their polar groups oriented towards the protein layers and their non-polar portions intermingling with each other [217]. The fatty acid tails in this region are largely of chain length C_{16} to C_{18}. A proportion of the fatty acid tails is unsaturated, which appears to afford stability to the membrane bilayer [218]. Saturated fatty acids afford porosity to lipoid layers for hydrophilic molecules. The ratio of 1 : 2 saturated to unsaturated fatty acids appears optimum for stability and porosity of the lipoid layer of membranes [218].

Sterols are present in the cell and internal membranes of most eukaryotic organisms [125,219] but the actual role of membrane sterol is not clear [220]. The membranes of some organisms may function with or without sterols (*Acholeplasma* or *Pythium* spp.) or in the complete absence of sterols (most prokaryotes) but for the majority of eukaryote cells the presence of sterol is essential to proper membrane function.

It has been mentioned previously that sterols were readily incorporated into lecithin or mixed phospholipid monolayers [194–196]. The area of sterol–containing films was smaller than that calculated for the separate components, indicating some kind of interaction between the two molecules [221,222]. Sterols have also been shown to reduce the phase transitions of pure lecithin dispersions [214]. It has been suggested that cholesterol modifies the fluidity of the hydrocarbon chains of the phospholipid molecules by disrupting the crystalline chain lattice of the gel phase and by inhibiting the flexing of the chains in the dispersed liquid-crystalline phase [221–226].

Cholesterol (13) has little ionic character and is shorter than membrane phospholipids [227]. In a mixed lipid layer, cholesterol would be expected to be accommodated towards the centre of the hydrocarbon region of the layer,



(13) Cholesterol

perhaps not even intruding into the ionic surface. This would provide increased space for the ionic groups of phospholipids to achieve a more stable orientation, whilst maintaining a close packing of the hydrocarbon structure. Increased close packing of the hydrocarbon structures may represent the main structural effect of the addition of cholesterol to a phospholipid layer [219]. A more specific complexing of phospholipid and cholesterol molecules has been proposed for the structure of myelin membranes by Finean [228] who suggested that sterols and phospholipids associate to form a compact unit, in which the Van der Waal's interaction would be very strong and in which ionic groups could be favourably disposed in relation to associated protein layers. In myelin membranes, the total phospholipid : sterol ratio was 1 : 1 and this ratio was found to be critical for cholesterol–phospholipid interaction. The spatial arrangement first suggested for this complex [228] was modified by Vandenheuvel [227] to improve Van der Waal's contacts and to accommodate unsaturation of the phospholipids (*Figure 3.3*). It will be seen from this figure that the phospholipid molecule is bent round the sterol molecule forming a bond with the C_3-terminal of cholesterol, the sterol molecule lying along one of the hydrocarbon chains.

The nature of the 3-hydroxyl group of incorporated sterol was important to the mobility and functional properties of membranes [229–231], e.g. 3β-hydroxyl groups decreased membrane permeability while 3α-hydroxyl groups had little effect [231]. It has been suggested that only 3β-hydroxyl groups bond with the carbonyl group of the lipid chain. The addition of 3β-hydroxysterols to lipid layers has been shown to have a profound retarding effect upon the kinetics of ionic transport [232]. It is well established that the permeability of phospholipid membranes diminishes markedly after cholesterol incorporation. This effect has been demonstrated for water [212], nonelectrolytes [152,178,179,233–235], anions [233,336] and cations [237]. Finean [219] proposed that the presence of sterol in membranes will produce a structure in which the liquid-crystalline lipid layers tend to be of minimum thickness. Butler, Smith and Schneider [238] suggested that sterols allow membranes to produce a preferred orientation of the bilayer structure in which the long axis of the lipid is orientated preferentially in the direction of the lamellar plane. The most acceptable hypothesis for the role of sterol was advanced by Chapman [239] who suggested

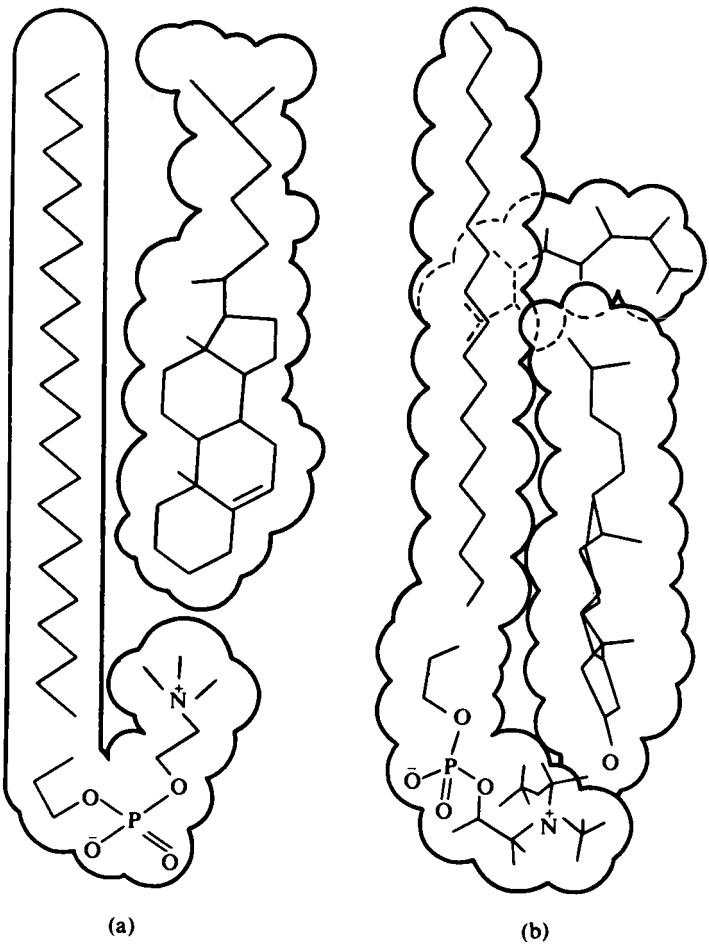


Figure 3.3. Suggested arrangements of the phospholipid–cholesterol complex according to (a) Finean [228] and (b) Vandenheuvel [227]. Reproduced from Finean [219]

that the presence of sterols caused the hydrocarbon chains of the phospholipid to be in an 'intermediate fluid condition', i.e. in the presence of sterol, the hydrocarbon chains of the phospholipids are in a state of conformational restriction intermediate to those in the gel and liquid-crystal phases. This allows some lattitude in the length and degree of unsaturation of the alkyl chain, resulting in a more ordered membrane. A model (*Figure 3.4*) has been proposed to illustrate the orientation of sterol esters in lipid bilayers [240,241] in which the phospholipid sterol ester is arranged such that the carbonyl group of the ester linkage was

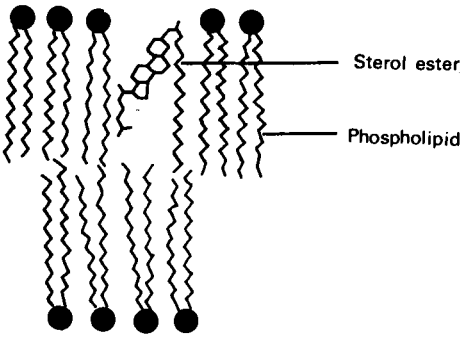
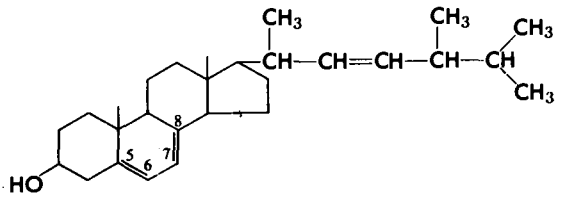


Figure 3.4. Orientation of sterol esters in lipid bilayers

orientated towards the polar regions of the bilayer while the sterol and fatty acid hydrocarbon chains penetrated the hydrophobic core of the bilayer.

The inner and outer components of the membrane bilayer have been shown to possess differing compositions and this is reflected in the different biological properties of the inside and outside of the cell membranes as reviewed by Bretscher [242]. X-Ray analysis of eukaryotic myelin membranes has demonstrated an asymmetric distribution of sterols, with almost double the sterol content in the outer bilayer leaflet as compared to the inner layer [243]. This asymmetry is reflected in *Figure 3.4.*

Although the composition and structure of mammalian (mainly erythrocyte and myelin) membranes has received extensive study, little attention has been paid to the membranes of yeasts, fungi and protozoa. Extraction of lipids from these organisms suggest similarities in overall structure, although cholesterol, which has an almost universal distribution in mammalian cells was replaced by other sterols, e.g. ergosterol (14) in yeasts and fungi [126]. The presence of



(14) Ergosterol

sterol creates order in the membranes of eukaryotes and it has been demonstrated that the fluidity of the membrane is important to the sensitivity of the organism to polyene antibiotics [185,244,245]. Those sterols which give maximal reduction in membrane permeability in synthetic bilayers, liposomes

derived from *A. laidlawii* and intact *A. laidlawii* cells [152,179,189,193,246] also showed optimal interaction with polyene antibiotics [146,147,150,152,179, 189]. When a sterol-ordered membrane comes into contact with a polyene antibiotic, preferential interaction occurs with those sterols which contribute greatest to the stability of the membrane explaining the dramatic changes in membrane permeability elicited by polyene antibiotics.

NATURE OF THE POLYENE DAMAGE SITE IN MEMBRANES

Synthetic membrane systems have given an insight into the mechanism of action of polyene antibiotics. Treatment of sterol-containing bilayer membranes with amphotericin B or nystatin reduced electrical resistance and increased ionic permeability but did not affect membrane stability. In contrast, filipin rapidly destroyed bilayers. The permeability of polyene-treated sterol-containing bilayers to hydrophilic nonelectrolytes increased in inverse proportion to molecular size, suggesting that damage sites were finite [246]. The transfer of ions and nonelectrolytes through lipid bilayers can be explained by the formation of pores or channels in which the polyol surface of the polyene molecule points inwards. Hladky and Haydon [247] suggested that aqueous pores may be present in bilayers, but if they were present they remained open for less than 100 ms. The electrical behaviour of nystatin- and amphotericin B-treated bilayers was consistent with the formation of non-static aqueous pores which coalesced and reformed continuously [202,203]. The size of such pores may be estimated by determining their reflection coefficients for a range of compounds of different equivalent radii (e.g. sucrose, raffinose, glucose, ribose, glycerol, acetamide, urea, etc.). It has been estimated that amphotericin B produces pores with a Stokes–Einstein radius of 7–10 Å [205], while those formed by nystatin were slightly smaller (4–7 Å) [201,248].

Several models for the structure of polyene–sterol pores in lipid bilayers have been presented [154,249–252]. They generally involve hydrophobic interaction between cholesterol and the unsaturated region of the polyene antibiotic, with possible hydrogen bond formation between the hydroxyl group of the sterol and the carbonyl group of the polyene ring.

Amphotericin B is the only polyene whose sterochemistry is completely understood [75]. Space-filling models of amphotericin B indicate that the polyene and polyol segments of the molecule are parallel and the molecule may be regarded as a rod-like structure some 20–24 Å long approximately equal to half the diameter of a lipid bilayer. *Figure 3.5* is based on the model proposed by Andreoli [251] for the amphotericin B–sterol complex in a bilayer. The hydrophobic components of the antibiotic molecule are situated in the membrane lying alongside the planar sterol ring. The figure shows hydrogen bonding

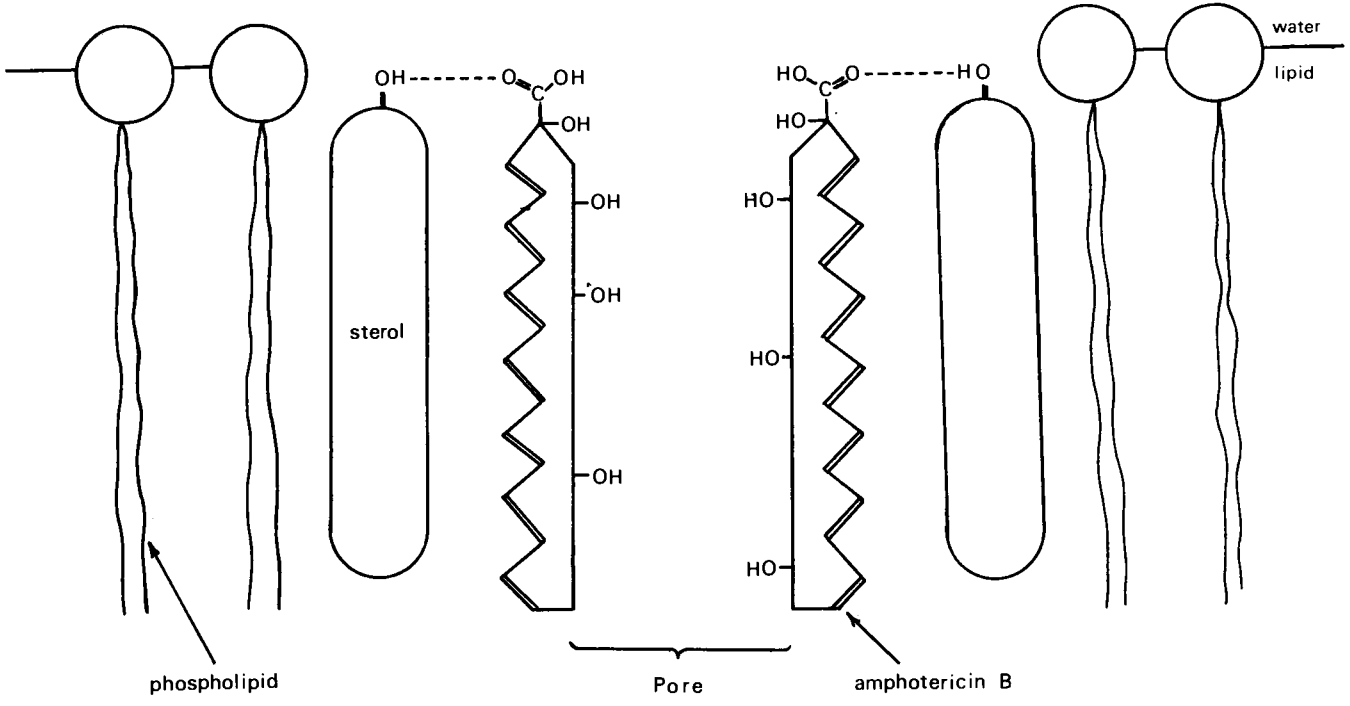


Figure 3.5. Postulated arrangement of a polyene–sterol pore in a lipid bilayer

between the 3β-hydroxyl group of the sterol and the C_{16}-carbonyl group of the antibiotic. Other possible interactions include bonding between the sterol 3β-hydroxyl group and the C_{17}-hydroxyl group, and hydrophilic interaction at the membrane–water interface. In such a structure, the mycosamine moiety would be located at the membrane–water interface. The unsaturated heptaene portion of the polyene macrolide ring may orientate towards the hydrophobic sterol ring, leaving the hydrophilic portion of the antibiotic to face the hydrophobic phospholipid chains. This is inherently unstable, so it is suggested that the sterol–polyene complexes come together to form a micelle, with the hydrophilic regions arranged in the centre, surrounded by the hydrophobic region [251, 252]. Hydroxyl groups line the pore, contributing to the observed polyene-dependent anion permselectivity [203,246]. The interactive forces between the polyene–sterol complex and adjacent phospholipid molecules will be minimal, i.e. the fluidity and hydration of the pore interior will be greater than in an unmodified membrane and may approach that of bulk water [249].

De Kruijff and Demel [154] also constructed space-filling models for cholesterol, distearoyl lecithins and a range of polyenes, enabling them to put forward a series of models capable of explaining many of the properties of polyenes.

The amphotericin B molecule (11) possesses hydrophilic groupings (i.e. seven hydroxyl groups and the ester linkage) aligned along one side of the molecule. On the opposite side is located the rigid hydrophobic double bond system. The length of the double bond system is about the length of a cholesterol molecule or a lecithin molecule (as measured from the charged phosphate group to the terminal methyl group). The cross-section of the amphotericin B molecule is nearly rectangular, while cholesterol approximates a pentagon. It is possible to visualise a polyene–sterol complex in which the sterol molecule fits partially into the ring of amphotericin B to give a combined radial angle of 23°. The complex can be extended to include an amphotericin B–sterol–amphotericin B–sterol complex with a radial angle of 45°. A further extension of this complex leads to a circular arrangement of 8 polyene–sterol units. The inside of such a unit would be hydrophilic, due to the hydroxyl groups of the amphotericin molecule, whereas the outside would be hydrophobic due to the conjugated double bonding. In such a structure the C_3-hydroxyl group of cholesterol and the charged groups of amphotericin B would be adjacent. The apolar backbones of the antibiotic and cholesterol would orientate parallel to the fatty acid chains of the phospholipids. The total length of the polyene–sterol complex approximates the length of the fatty acid and glycerol moieties of the phospholipid molecules, so that the sterol–polyene complex forms a half pore through the lipid bilayer. Such half pores may float independently in the lipid film; a full pore only being formed when two half pores, from each side of the membrane approach and bond.

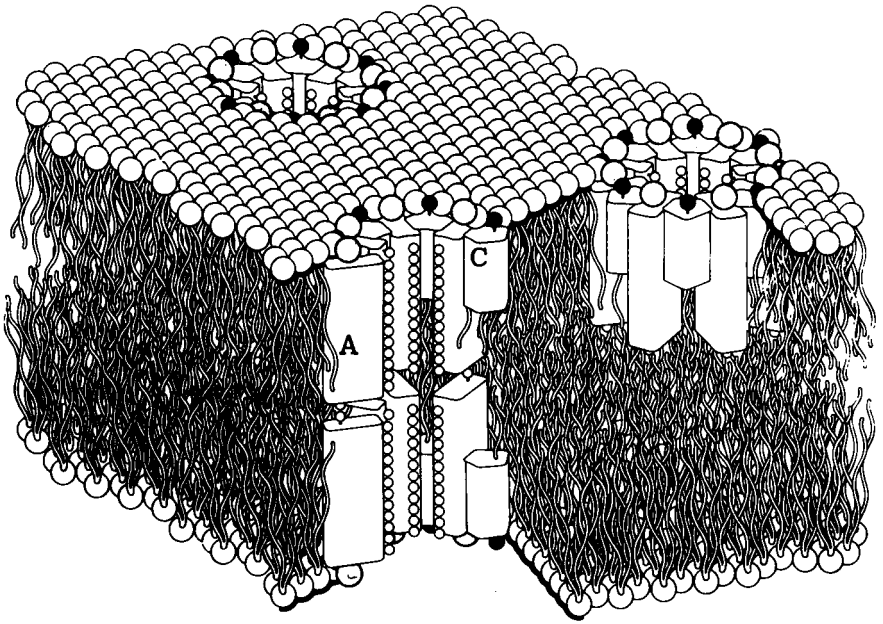


Figure 3.6. Schematic representation of pores formed by amphotericin B (A) and cholesterol (C) in a lipid bilayer, showing in cross-section the interior of a conducting pore formed from two half pores; from de Kruijff and Demel [154]

Figure 3.6 is a schematic representation of pores formed by amphotericin B on combination with cholesterol in a lipid bilayer. The figure shows in cross-section the conducting pore formed by two half pores. This model and the other similar models [249,251–253] agree with much of the experimental data concerning amphotericin B:

(a) Amphotericin B interacts with cholesterol in such a way that sterol is drawn away from its interaction with other lipids [146,147].
(b) The amphotericin B–cholesterol interaction is hydrophobic [147,153,173].
(c) The stoichiometry of the amphotericin B–cholesterol interaction approaches unity [153].
(d) The observed polyene-induced permeability changes are dependent on antibiotic concentration and indicate that between 5 and 10 amphotericin B molecules are necessary to form a conducting pore [173,203].
(e) Permeability studies suggest that glucose molecules only pass very slowly or not at all through such pores [173,203,246], i.e. the pore diameter approaches the diameter of the glucose molecule.

(f) An intact polyene molecule is necessary for the construction of such models. Hydrolysis of the lactone ring or saturation of the chromophore makes pore formation theoretically impossible. No pore has been demonstrated with such modified polyenes [203].

The structure of nystatin (8) is closely related to that of amphotericin B (11) except that in the nystatin molecule the double bond system is interrupted by saturation of the bond separating the tetraene and diene chromophores. This would allow the bending of an otherwise rigid structure. A similar structure to that of the amphotericin B–sterol pores has been proposed for nystatin in sterol–lecithin bilayers [154,203,249]. The permeability characteristics of nystatin pores closely resembled amphotericin B pores and it was even possible to form mixed pores when nystatin was added to one side of the bilayer and amphotericin B to the other [209].

Nystatin and amphotericin B induced a cation-selective conductance when added to one side of a lipid bilayer but induce anion-selective conductance when added to both sides [203]. The antibiotic concentrations required to produce permselective changes on one side of the bilayers were much greater than those required to demonstrate activity when introduced on both sides of the membrane [154,254]. The two-sided effect resulted from the formation of aqueous pores through the bilayer due to the combination of two half pores. The one-sided effect resulted from the changes in permeability induced by half pores alone.

When the aromatic heptaene, candicidin, was added to both sides of a bilayer under similar conditions, a cation-selective pore was produced (in contrast with the anion-selective pore produced by nystatin and amphotericin) [203]. This antibiotic forms a different type of pore, as unlike nystatin or amphotericin B it was equally effective when only added to one side of the membrane [255].

The tetraene etruscomycin (7) possesses a smaller macrolide ring than either amphotericin B- or nystatin containing a four-carbon hydrophobic chain attached to the macrolide ring. This hydrophobic tail is thought to play a vital role in the effect of etruscomycin on bilayers since pimaricin, which has an identical structure excepting that this chain is absent, showed no effect on bilayer permeability [147]. The orientation of the charged carbonyl and mycosamine residues are identical with those of nystatin and amphotericin B. Half pores for an etruscomycin–cholesterol complex have been constructed [154] and have a total length, including the hydrophobic tail, equal to the length of the amphotericin or nystatin pore. It has been suggested that complete etruscomycin pores form when two half pores oppose one another stabilized by hydrophobic interaction between the hydrophobic tails at the end of each half pore.

Pimaricin (6) does not possess such a hydrophobic tail and did not alter the

permeability of *A. laidlawii* or egg lecithin liposomes [173]. Pimaricin can therefore only form half pores since the total length of a pimaricin–sterol complex would be less than half the bilayer thickness [154].

In contrast with other polyenes, filipin (8) possesses neither carbonyl or amino groups but contains a hydrophilic alkyl chain lying adjacent to the hydrophilic hydroxylated portion of the molecule. This structure makes it impossible to construct pores for a filipin–sterol complex with an orientation perpendicular to the plane of the bilayer, but the absence of a bulky mycosamine moiety allows space-filling models of filipin and cholesterol to be packed in a parallel array. Such arrangements can then be combined in parallel to form extensive aggregates. One side of such an aggregate would be made hydrophobic by the presence of the pentaene chromophore and the opposite side hydrophilic due to the polyol surface. Two such planar aggregates could associate to make a 'sandwich', with the hydrophilic regions in the centre and the hydrophobic outside of the 'sandwich' interacting with cholesterol molecules (*Figure 3.7*). Such a structure bears little resemblance to a pore and it is suggested that such aggregates merely disrupt the membrane. There is some experimental evidence to support such a model:

(a) Filipin–sterol interaction is stronger than phospholipid–sterol interaction since cholesterol is withdrawn by filipin from its interaction with phospholipids [146,147,153].
(b) The filipin–sterol interaction is hydrophobic [147,153].
(c) Freeze-etch electron microscopy shows filipin–cholesterol aggregates (diameter 150–250 Å) lying in the hydrophobic core of the bilayer [256,257].
(d) The effect of added cholesterol on the UV spectrum of polyene antibiotics is maximal for filipin, even though it possesses a chromophore intermediate in length between that of amphotericin B and pimaricin [146,147]. Assuming that the change in UV absorption spectrum result from an orientation of the sterol polyene complex in which the adjacent chromophores influence one another, then filipin produces the greatest effect on the spectrum because the chromophores are closest in the filipin–cholesterol complex [147].
(e) Filipin rapidly lysed *A. laidlawii* and mammalian erythrocyte membranes and, from comparison of the rate constants for the formation of filipin–cholesterol aggregate and induced ion leakage, it was suggested that the formation of complexes preceded membrane disruption [153].
(f) The interaction between filipin and bilayers must differ markedly from the interaction of amphotericin B and nystatin with bilayers, since unlike these antibiotics filipin lyses bilayers so rapidly that pore formation cannot be detected [199,210].

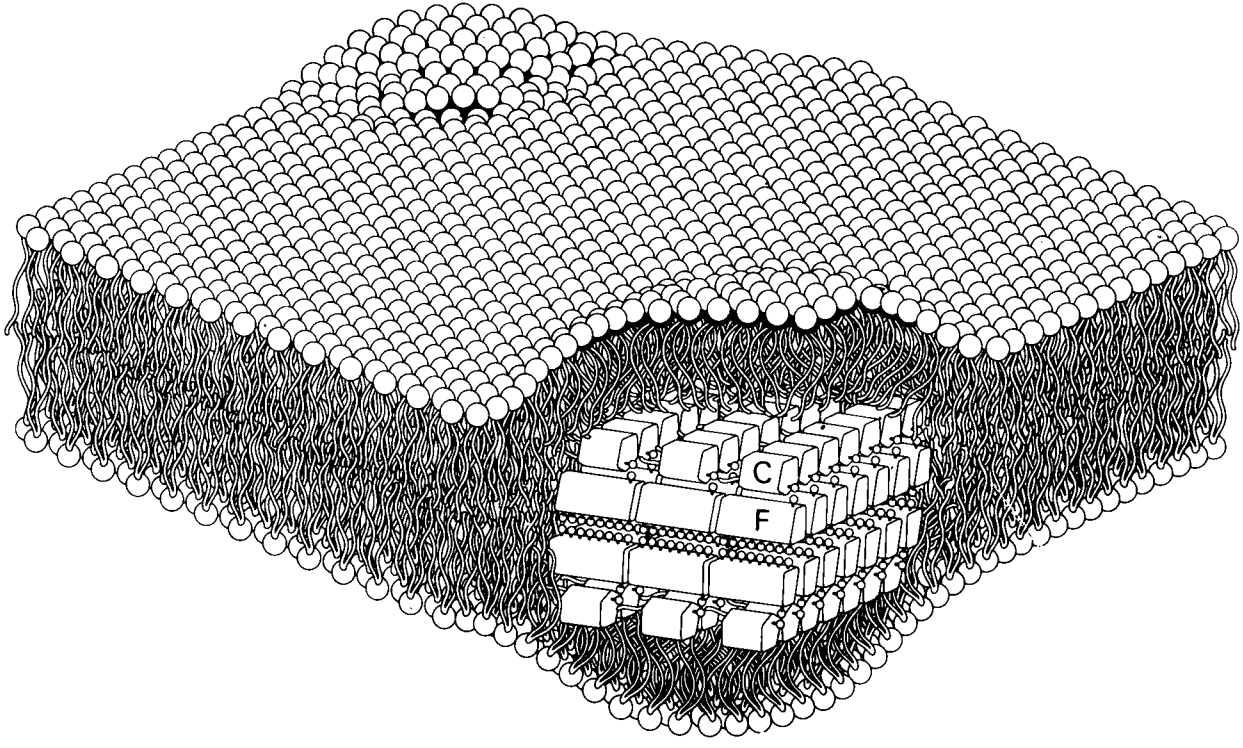


Figure 3.7. Schematic representation of the cholesterol (C)–filipin (F) complex in a lipid bilayer, showing a cross-section through part of a filipin–cholesterol complex; from de Kruijff and Demel [154]

The models proposed for the polyene bilayer sterol pores are very attractive, but, although they go some way towards explaining the action of polyenes on membranes, the validity of such models, especially when applied to natural membranes, have been questioned. Several workers have suggested that although sterols are necessary for polyene bilayer interaction their presence *per se* do not confer sensitivity. It appears that the ordered state in the bilayer resulting from the presence of sterol ultimately confers polyene sensitivity [185,244,245]. It has also been suggested [253] that the one-sided and two-sided channels are not identical (in contrast with the de Kruijff and Demal model illustrated). It will be remembered that to produce a pore through a bilayer membrane it requires antibiotic molecules to interact from both sides of the membrane. When whole cells are treated with polyene antibiotic the membrane can only be attacked from the 'outside'. It seems unlikely, but not impossible, that sterol–polyene complexes can 'flip' from one membrane leaflet to the other [248], suggesting that such simple sterol–polyene pore cannot completely explain the polyene-induced permeability changes observed in whole cells. It is likely that the proposed models will be modified to reflect roles for sterols, phospholipids and perhaps even proteins, but for all their limitations they have provided for the first time a rational interpretation of differing behaviour of individual polyenes in bilayers.

Attempts have been made to observe physical changes in membrane structure after polyene treatment. Negative staining electron microscopy has revealed areas of disorder approximately 120 Å in diameter in filipin-treated sterol-containing lecithin liposomes [170,233]. Similar 'pits' (150–250 Å diameter) were shown in freeze-etch preparations of erythrocytes, sterol-containing liposomes and sterol-supplemented *A. laidlawii* cells [256]. It was shown that the 'pits' were not 'pores', i.e. they did not penetrate the membrane. The 'pits' appear to represent areas of disorganised aggregated material, possibly the polyene–sterol complexes. It has been suggested that filipin induces lamellar to micellar phase changes in membrane structure. Aggregates were not formed by filipin in membranes which did not contain sterols [256]. Examination of freeze-etch electron microscope preparations of filipin-treated sterol-containing liposomes or erythrocyte membranes [257,258] revealed structural alterations including pits (diameter 150 Å), doughnut-shaped craters (overall diameter 400 Å, inside crater diameter 150 Å) and protrusions (300 Å long). No explanation of the course of development of these structures was given but it was demonstrated that none of the alterations in membrane structure penetrated the membrane. The development of 'pits' has also been reported in egg lecithin–sterol vesicles treated with filipin III [155]. Filipin III also produced 'pits' in the cillary membrane of the protozoan *Tetrahymena pyriformis* [155]. Saponins which are known to extract sterols from membranes also produce 'pits' in sterol-containing membranes [259, 260]. Saponin-induced 'pits' were distributed evenly over the membrane surface,

representing sites from which cholesterol has been drawn from solution. Similar 'pits' have been demonstrated in immune-lysed erythrocytes [260].

Freeze-etch preparations of sterol-containing liposomes or erythrocyte membranes treated with amphotericin B did not reveal 'pits' [256]. The postulated amphotericin B–sterol pores would be too small to be seen by this technique. However, freeze-etch studies of amphotericin B-treated plasma membranes isolated from *Epidermophyton floccosum* [261], a fungus pathogenic to man, has revealed depressions (or craters) and aggregates of membrane-associated particles (85 Å in diameter). The areas of disruption did not traverse the membrane.

SEQUENCE OF ANTIBIOTIC ACTION OF POLYENE ANTIBIOTICS

Polyene antibiotics are essentially hydrophobic molecules, even though the molecule possesses a hydrophilic region. In aqueous solution, they exist as a lyophobic sol of polymolecular aggregates but because the antibiotic has greater affinity for lipoid surfaces polyene molecules will leave the aggregate if such a surface is present. Uptake of polyene antibiotics is rapid [77,138,162]. It has been suggested that yeast cells present two binding sites to the antibiotic [71, 123,138,162,168]. The first was located in the cell wall and as the cell walls of yeasts are sterol-free [126] this site is presumed to be nonsteroid [162,168]. This wall site bound a large proportion of the total antibiotic taken up by the cell and it appeared that the binding requirement of this site must be fulfilled before the antibiotic can reach the membrane [168]. Apparently antibiotic absorption by the cell wall has little effect on microbial metabolism.

Given that the polyene-binding capacity of the cell wall has been satisfied (obviously this wall binding capacity only applies to eukaryotes with cell walls but not to fungal protoplasts, protozoan or mammalian cells) the polyene molecule will reach the cell membrane.

Membranes consist mainly of phospholipid molecules orientated with their hydrophilic phosphate group lying at the aqueous interface and the hydrocarbon chain orientated towards the hydrophobic membrane core. The sterol molecules present in eukaryotic membranes orientate so that the 3β-hydroxyl group of the sterol ring lies at the aqueous interface. It is thought that the first contact between polyene and the sterol occurs at this 3β-hydroxyl group. When the antibiotic molecule reaches the membrane, the strong affinity of the antibiotic for the sterol drags the polyene molecule into the membrane, so that the hydrophilic unsaturated polyene chromophore lies alongside the sterol molecule. This not only creates instability in the membrane by breaking the phospholipid–sterol interactions which stabilise the membrane, but also introduces the polyol region of the polyene into the membrane. This intrusion of a hydrophilic chain

into the hydrophobic membrane may be sufficient to account for all the observed polyene-induced permeability changes in membranes. Alternatively, the sterol–polyene complexes may come together to form aggregates with the hydrophilic regions of the antibiotic molecules arranged in the centre, surrounded by a hydrophobic region (i.e. to form half pores as have been described for polyene-treated bilayers). Whatever the mechanism, the polyene–sterol interaction is sufficient to disorganise the membrane to such an extent that it can no longer present a permeability barrier between cell contents and the environment.

It has been claimed that there are two different mechanisms controlling the binding between amphotericin B and the membrane of the yeast *S. cerevisiae:* weak reversible binding which occured at 0°C, at higher temperatures or even in the presence of metabolic inhibitors, and a stronger irreversible binding which could not occur at low temperatures and was inhibited when energy metabolism was blocked [262]. Strong binding correlated with antifungal activity. It was proposed that weak binding, representing non-lethal binding to the outside of the membrane, was followed by lethal stronger binding to the hydrophobic core of the membrane (in accord with the model of de Kruijff and Demel [154].). However, this study fails to take into account the known binding of polyenes by yeast cell walls [71,123,138,162,168] and the 'weak' binding may in fact represent binding to the cell wall and the 'strong' binding interaction with the yeast membrane.

It has been suggested that initial uptake of polyene antibiotics was an energy-requiring process [77,122,162] but this is somewhat at variance with the results of Kinsky [109,132,160]. Polyene uptake is restricted at low temperatures and this may reflect the reduced thermal mobilities of groups concerned with antibiotic uptake, especially lipids, at low temperatures. Alternatively, metabolic energy may be required to maintain binding site accessibility or to move antibiotic molecules to the active site. When energy production was diminished by denial of all metabolisable substrates [77,162,263] or adding metabolic inhibitors [122,162], polyene binding and antifungal effects are reduced.

Filipin inhibited anaerobic and aerobic oxidation of glucose by *S. cerevisiae* but had no effect on the oxidative capacity of cell-free homogenates [141]. The antibiotic reduced the dry weight of the yeast with the loss of nitrogen and phosphorus from the cell. This was the first indication that the observed effects of polyenes on fungal metabolism were a consequence of altered cellular permeability and the fungicidal effect was the result of the loss of vital cytoplasmic components.

Several marker molecules have been used to study membrane damage in micro-organisms, including ions, sugars and 260 nm-absorbing material (predominantly amino acids and nucleotides) [264]. The sensitivity of such tests is inversely related to the molecular weight of the marker [265,266]. Cells actively

take up potassium ions from the environment against a concentration gradient and consequently contain relatively high potassium concentrations. The leakage of potassium ions from microbial cells may be regarded as one of the first indications of membrane damage [267]. The use of ion-selective K^+-electrodes allows *in situ* rapid monitoring of antibiotic-induced K^+ fluxes in microbial suspensions. Using this technique, K^+ leakage from polyene-treated yeasts has been detected within 2 min of antibiotic application. Polyene-induced ion fluxes were completed within 20 min [268,269]. Yeast protoplasts or mammalian cells leak K^+ ions almost instantaneously after polyene treatment [268,269], reflecting the easier access for the antibiotic to the sterol in the cell membrane due to the lack of a cell wall structure. The time taken for the antibiotic to cross the cell wall before damaging the membrane (i.e. the time between drug application and the onset of K^+ leakage) was considered too great to represent the simple diffusion time across the cell wall and was thought to represent the time taken for the wall binding capacity to be satisfied. The delay before the onset of potassium-induced K^+ leakage in *C. albicans* is not constant but increases with the age of culture [268,269] and the polyene binding capacity of the yeast cell wall [268].

Growth inhibitory concentrations of polyene antibiotics alter the selective permeabilities of eukaryote membranes. Leakage of potassium ions, carboxylic and amino acids and phosphate esters have been reported [101,106,107,267–271]. Larger molecules, e.g. proteins and nucleic acids, were not lost from the yeast [106,107].

Potassium leakage is more important in the mode of action of polyene antibiotics than merely an indication of membrane damage. It has been reported that antifungal concentrations of filipin, pimaricin, rimocidin, fungichromin, nystatin, candidin, candicidin, amphotericin B, amphotericin B methyl ester and *N*-acetyl amphotericin B all produce potassium leakage from yeasts [267–271]. It has been shown that approximately 90% of the total free potassium pool of the yeast was lost on polyene treatment [268]. Intracellular potassium has many functions in the fungal cell. Potassium ions are co-factors of 3 distinct steps in the glycolytic pathway [272–274] and have been implicated in fatty acid oxidation, syntheses of ribonucleotides, syntheses of glutathione and other cellular reactions [275]. Potassium-depleted yeasts show changed respiratory activity and low growth rates [276].

It has been demonstrated that polyene-induced K^+ leakage was closely followed by inhibition of yeast glycolysis [277]. In the case of certain polyenes, this inhibition of yeast glycolysis could be reversed at neutral pH by extracellular concentrations of NH_4^+ or K^+ ions. In contrast, other polyenes were found to cause irreversible inhibition of yeast glycolysis. This led Lampen and Arnow [106,107,278] to divide the polyene antibiotics into two distinct groups on the basis of their antibiotic action: the small-ring polyenes, including filipin,

etrusconiycin, pimaricin and rimocidin, and the large-ring polyenes, e.g. amphotericin B, candidin, candicidin and nystatin. The small-ring polyenes produced such dramatic changes in membrane structure that inhibition of glycolysis was not reversed; they induced a loss of phosphate and sugars and caused lysis of protoplasts and erythrocytes. The large-ring polyenes induced more subtle changes in membrane structure and produced considerable specificity in the observed effects. Inhibition of glycolysis was reversed by extracellular concentrations of K^+ or NH_4^+. The use of higher nystatin concentrations (>10 μg/ml), at neutral pH, also inhibited yeast glycolysis but this was not reversed by the presence of K^+ or NH_4^+ ions. When adenosine triphosphate, nicotinamide dinucleotide, thiamine pyrophosphate, pyruvate and Mg^{2+} ions were added, glycolysis was restored. Higher antibiotic concentrations must also allow the leakage of other cytoplasmic components necessary for glycolysis. It is important to note that addition of these ions did not prevent antibiotic absorption or increase the number of survivors [279–283], i.e. other cytoplasmic components necessary for fungal viability but unnecessary for glycolysis were also lost from the cell or more likely the destruction of membrane selective permeability was so complete that death was inevitable. Extracellular K^+ or NH_4^+ merely prevented the diffusion of these ions from the cells and allowed glycolysis to proceed. Large-ring polyenes produced lysis of protoplasts only at high concentration after prolonged exposure, if at all (*Table 3.5*). However, it would be wrong to think that polyenes neatly divide into two groups. The classification based on ring size was developed in part to reconcile the tetraene chromophore of nystatin with its antibiotic properties. However, if nystatin is regarded as a 'degenerate' or modified heptaene, then a classification based on the size of the chromophore can be proposed. The antimicrobial properties of polyenes increase with the size of the chromophore (filipin $\ll$ luscensomycin = pimaricin $<$ candidin = nystatin $<$ amphotericin B $<$ candicidin). The pentaene filipin showed the greatest membrane-disrupting activity in terms of erythrocyte or fungal protoplast lysis, inter-

Table 3.5. OBSERVABLE ANTIBIOTIC EFFECTS OF FOUR POLYENES

	Filipin	*Nystatin*	*Amphotericin B*	*Candicidin*
Potassium leakage	+	+	+	+
K^+ or NH_4^+ reversal of glycolysis inhibition	–	–	+	+
Phosphate leakage	+	+	±	±
Erythrocyte lysis	rapid	+	+	±
Yeast protoplast lysis	rapid	+	±	–
Antifungal activity	+	++	+++	++++

action with liposomes, monolayers or bilayers and had the greatest affinity for sterols. Other polyenes showed diminished membrane active properties in the order filipin ≫ amphotericin B > luscensomycin = pimaricin > nystatin > candicidin. Although filipin has the greatest destructive effect on eukaryote membranes its antifungal effect is lower than that shown by other polyenes. The ionic neutrality of filipin gives it certain properties not shared by the other polyenes.

Polyene treatment also caused loss of cellular magnesium [283–285]. It has been shown that magnesium leakage in candicidin-treated yeasts occurred much later than that of potassium [284]; in the first fifteen minutes after antibiotic treatment, magnesium leakage was slight but then was followed by a rapid, antibiotic concentration-dependent release of magnesium [284]. Many enzymic activities of fungal cells depend upon the presence of magnesium ions [275] and the loss of a major part of cellular magnesium would rapidly bring metabolism to a halt.

Calcium ions were lost from yeasts upon treatment with lethal levels of candicidin [283] and this loss could have profound effects upon cell metabolism. Calcium ions are regulators for glycogenolysis and gluconeogenesis and are involved with the control of desoxyribonuclease, phospholipase and proteolytic enzymes [286].

Pre-treatment with calcium or magnesium ions protected membranes from polyene damage [270,283,287–289]. Calcium has been shown to complex with polyene molecules and this may lower the effective antibiotic concentration. Calcium and magnesium ions have been shown to form complexes at the C_3 β-hydroxyl group of sterols [283,290]. It is interesting to note that this hydroxyl group also plays a role in the formation of phospholipid sterol esters [219] and has been implicated in the formation of the sterol–polyene complexes [145]. The uptake of divalent ions by this function may create steric hindrance to subsequent antibiotic uptake thereby reducing membrane damage [283].

It has been reported that between 10 and 20 min after candicidin treatment of yeasts that protein syntheses slowed and then ceased. Protein synthesis slowed not as a result of energy deficiency, as ATP was abundant in polyene-treated cells at this stage, but as a direct result of reduced potassium levels [287]. Similar reductions in protein syntheses have been observed in *E. coli* mutants with defective potassium transport systems [291]. Upon treatment with levels of nystatin that did not affect yeast respiration, the uptake of glycine was rapidly reduced and ceased within 20 min of antibiotic application. Low nystatin concentrations did not produce leakage of amino acids into the environment, suggesting that nystatin at high concentrations damaged the cell membrane to such an extent that all normal uptake processes ceased [292].

The loss of potassium ions, and to a lesser extent calcium and magnesium ions

from the yeast cell, results in a change in internal charge. As the selective permeability properties of the membrane have been altered by the antibiotic, it could be expected that lost cations would be replaced (at a pH less than 7) by protons from the environment. The transfer of protons from the environment to the cytoplasm of polyene-treated yeast has been demonstrated [293]. This inflow of protons resulted in acidification of the yeast cytoplasm. The pH of the cell cytoplasm of *C. albicans* fell from 6.12 to 5.20 on treatment with lethal levels of candicidin [293]. However, this degree of acidification was too great to represent a simple K^+–H^+ exchange as postulated by Lampen [106,107]. At low pH (<5.8) inhibition of glycolysis by nystatin could not be reversed by addition of a mixture of cations and cofactors [279–281]. The lower pH by increasing the external proton concentration may lead to more rapid internal acidification.

The internal acidification of polyene-treated yeast cells took up to 4 h and resulted in precipitation of the yeast cytoplasm. The pH change induced in yeast cells by lethal antibiotic levels was sufficient to cause a marked increase in the optical density of yeast cell sap *in vitro*. Yeast suspensions exhibited concentration-dependent increases in optical density when treated with nystatin or candicidin [270,294]. The increase in optical density resulted from an increase in the refractive index of the individual yeast cells and reflected precipitation of certain cytoplasmic components [294]. Light microscopy has demonstrated increased granularity in yeast treated with lethal levels of nystatin. Marked granularity and loss of organelle definition has been observed by electron microscopy in yeasts treated with filipin, amphotericin B [295,296] hamycin [296] and candicidin [293,294]. The effects of filipin and amphotericin B upon yeast morphology were similar but filipin-treated yeasts showed cytoplasmic precipitation and organelle loss much more rapidly than did yeasts treated with amphotericin B [295]. *Plate 3.1* shows the effect of lethal levels of candicidin upon the morphology of *C. albicans*.

The point at which the fungal cell can be considered dead is debatable [297]. It is not possible to reverse the lethal action of polyenes once sufficient antibiotic molecules have combined with the cell membrane. Lethal levels of nystatin brought glycolysis of *S. cerevisiae* to a halt within 40 min [298] and 95% of the yeast cells were not viable after 30 min. Cell death inevitably follows destruction of membrane permeability. Whether the observed changes in cell metabolism, composition or morphology are causes or symptoms of death is unclear: the sequence of antibiotic action in organisms other than fungi has received little attention.

Figure 3.8 summarises some of the observable effects occurring in *C. albicans* after treatment with a lethal level of candicidin. Leakage of small molecular weight material (represented by potassium ions) follows closely on antibiotic uptake. Precipitation of the yeast cytoplasm (as represented by a rise in cell

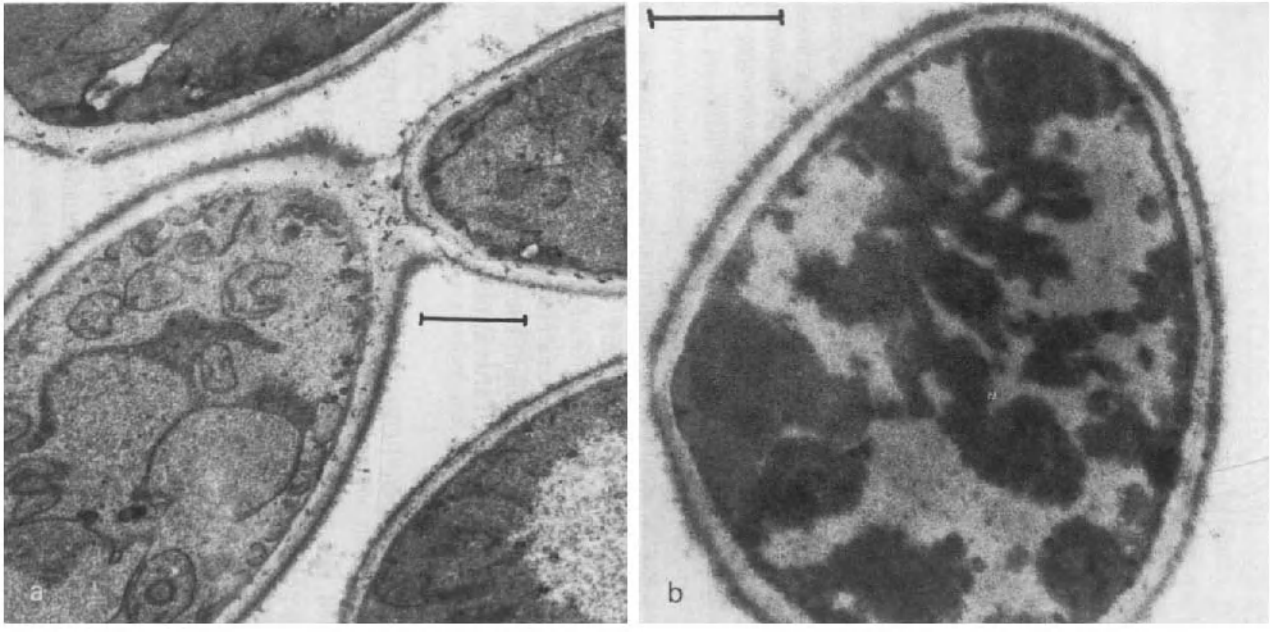


Plate 3.1. Electron micrographs of C. albicans *fixed with potassium permanganate and stained with lead citrate. (a) Untreated yeasts. (b) Treated with 10 μg candicidin/ml for 1 h (bar = 0.5 μm)*

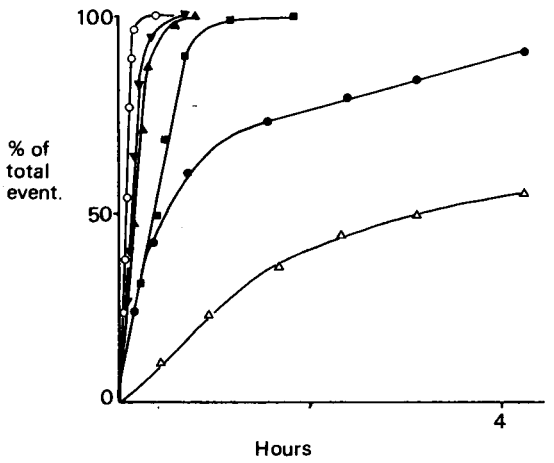


Figure 3.8. Effect of 25 μg candicidin/ml upon a standard suspension of C. albicans *NCTC 713. (○) Candicidin uptake, (▼) candicidin-induced K^+ leakage, (▲) candicidin-induced change in cell refractive index, (■) percentage yeasts killed, (●) candicidin-induced inhibition of O_2 uptake and (△) candicidin-induced proton uptake*

refractivity) increases up to 1 h after candicidin treatment. Within 30 min of candicidin treatment, growth ceases and the survival rate drops to 0.01%. Oxygen uptake continues in the presence of candicidin for several hours suggesting that cellular enzyme systems are unaffected by candicidin and function long after the apparent death of the organism. The yeast continues to take up protons from the environment for up to 4 h after antibiotic treatment.

RESISTANCE OF FUNGI TO POLYENE ANTIBIOTICS

It is often stated that resistance to polyene antibiotics does not occur [103, 299–301]. It is perhaps more true to say that although yeasts and other fungi are capable of giving rise to polyene-resistant strains, polyene resistance has not become a clinical problem, even after twenty years of therapeutic use. This is fortunate, for an increase in resistance of twenty-fold would be sufficient to render many strains of pathogenic and opportunistic fungi resistant to antibiotic concentrations that can be achieved in clinical lesions by conventional polyene therapy.

In vitro polyene resistance has been demonstrated in yeasts [301–311], dermatophytes [312,313], *Aspergillus* sp. [314] and *Neurospora* sp. [315]. Resistance to polyenes does not confer resistance to non-polyene antifungal

agents but cross-resistance between polyenes is almost universal [300,301,310, 316,317]. Many yeasts are capable of induced or spontaneous mutations to give polyene-resistant straints. Single-step nystatin-resistant variants, at a frequency of approximately 1 in 10^7, have been demonstrated in *C. albicans* [318]. Single-step polyene-resistant fungi have been induced by UV radiation [311, 319] and by mutagenic agents including ethyl methane sulphonate [309], nitrous acid [315], and *N'*-methyl-*N'*-nitrosoguanidine [311]. Multistep resistance, induced by growing fungi in successive increasing antibiotic concentrations, have also been described [302–304, 320–322]. Most cases of polyene resistance, whether single or multistep, were accompanied by quantitative or qualitative changes in the sterol component of the fungal cell. Polyene-resistant fungi, on which sterol analysis has been carried out, can be divided into three groups:

(1) Those polyene-resistant mutants which contained either decreased ergosterol (as compared with the wild type) or none at all, (including the *S. cerevisiae* strains designated *nyr*-1, *nyr*-2, *pol*-2, *pol*-4 and *pol*-5 by Bard [323] and Molzahn and Woods [308,309]; the *N. crassa* strains I, II and III of Grindle [315] and the resistant strains of *C. albicans* studied by Athar and Winner [301]). Presumably this group achieved resistance by possessing fewer potential polyene binding sites.

(2) Those strains in which some of the ergosterol has been replaced by another sterol, the replacement sterol possessing a lower polyene affinity than ergosterol. This group includes the *S. cerevisiae* mutants designated *nys*-1, *nys*-3 (renamed *pol*-1 and *pol*-2 by Molzahn and Woods [309]) and *nyr*-15 [308], the *ole*-2-1, *ole*-2-2, *ole*-3 and *ole*-4 petit mutants of *S. cerevisiae* [323], ergosterol-deficient yeast mutants [324], *Aspergillus fennelliae* mutants [314], resistant *Cryptococcus neoformans* [325] and resistant mutants of *N. crassa* [326]).

The major sterol isolated from resistant fungi may be novel; e.g. $\Delta^{8,22}$-ergostadien-3β-ol [327] has been shown to be the major sterol of nystatin-resistant *S. cerevisiae* mutants described by Molzahn and Woods [308,309]. Alternatively, biosynthetic precursors of ergosterol may constitute the major sterol in resistant fungi. Mutants of *S. cerevisiae* showing low levels of nystatin resistance were found to contain large amounts of 5,6-dihydroergosterol, the immediate precursor of ergosterol [329]. Mutants resistant to higher nystatin concentrations contain a mixture of earlier precursors. Ergosterol-requiring nystatin-resistant mutants were found to contain increased amounts of lanosterol and other minor sterols and decreased ergosterol levels as compared with the wild type. It was suggested that in such mutants the removal of the C_{14}-methyl group of lanosterol was blocked [324]. Four nystatin-resistant mutants of *S. cerevisiae* (*pol* 1,2,3 and 5) have been shown

to contain certain metabolic blocks preventing normal ergosterol biosynthesis (including C_{24} transmethylation, Δ^8 to Δ^7 isomerase and 22,23-dehydrogenase activity) [329]. Identification of the sterol content of polyene-resistant mutants of *Aspergillus fenneliae* revealed that $\Delta^{7,22}$-ergostadien-3β-ol, and $\Delta^{7,22,24(28)}$-ergostatrien-3β-ol were the main sterols present, while ergosterol (a $\Delta^{5,7}$ sterol), the major sterol present in the wild type, was lacking. The mutants appeared to have lost the ability to dehydrogenate the C_5–C_6 bond of the sterol ring and to a lesser extent the ability to reduce the C_{24}–C_{28} bond [330]. The *nys*-3 (or *pol*-3) mutants of *S. cerevisiae* [308, 309] were found to produce several Δ^7 and Δ^8 sterols but relatively little $\Delta^{5,7}$ sterol [308,309,327–329]. Mutants which produced Δ^8 sterols were more resistant than mutants containing Δ^7 sterols. This suggests that $\Delta^{5,7}$ sterols are more effective in binding polyene than Δ^7 sterols, which in turn are more effective than Δ^8 sterols.

(3) A small group of *C. albicans* mutants, induced by *N'*-methyl-*N'*-nitrosoguanidine, were found to contain more ergosterol than the wild type [311] and it has been suggested that this additional sterol is either re-orientated or masked in some way so that polyene binding is lowered. The observation that sterol incorporation *per se* does not always increase the polyene sensitivity of liposomes, i.e. it is the membrane state induced by sterol, not the total quantity which confers polyene sensitivity, which may provide a partial explanation of this case.

Several observations fall outside the classification. Capek and Simek have claimed that three dematophyte fungi (*Trichophyton mentagrophytes, T. rubrum* and *Microsporium gypseum*) produce a nystatin-destroying enzyme [331,332]. It has also been claimed that nystatin resistance exhibited by tetraploid *S. cerevisiae* cells as compared with haploid cells of the same species was due to differences in the surface area : volume ratio, not an intrinsic biochemical difference [333]. This hypothesis has been shown not to apply to other polyene-resistant yeasts [334]. A study on the resistance of *C. albicans* isolates and mutants indicated an additional type of nystatin resistance [335]. Resistance of several strains arose from changed or reduced sterol levels as previously described. The remaining strains achieved resistance through a novel mechanism, whereby sterol content was unaltered and antibiotic was found to bind to the cell membrane. It appeared that some later, but unspecified, step in the fungicidal action of the antibiotic was prevented.

Relatively few reports exist of *in vivo* acquisition of polyene resistance and those cases reported only possessed low levels of resistance (three to four times the minimum inhibitory concentration) [336,337]. Examination of large numbers of clinical isolates of *C. albicans* has failed to demonstrate resistance to

amphotericin B and nystatin [301,325,338] but many of these isolates could develop resistance *in vitro*. It has been reported that clinical isolates of *C. albicans* rapidly lost any polyene resistance they possessed when grown on normal laboratory medium [138].

The failure of pathogenic or opportunistic fungi to develop significant resistance to polyenes suggests that any major change in membrane structure, i.e. a reduction in sterol levels, or the substitution of one sterol for another, constitutes a lethal mutation, in that such fungi show reduced metabolic activity, declining growth rates and diminished virulence [301–304,311,322,339,340].

A series of papers [305–310,323] examined the genetics of polyene resistance in *S. cerevisiae* and suggested that nystatin resistance was controlled by three recessive genes and two dominant modifiers. The genes that gave rise to the *nys*-1, *nys*-2 and *nys*-3 mutants differed markedly in their ability to give cross-resistance to filipin and amphotericin B, *nys*-1 confered resistance to amphotericin B and nystatin but not to filipin, while *nys*-2 and *nys*-3 confered resistance to nystatin and filipin but made the yeasts hypersensitive to amphotericin B. Similar discrepancies in polyene cross-resistance have also been described for *C. albicans* [311]. Also described were cytoplasmic polyene-resistant mutants which gave complete cross-resistance to all polyenes tested [305].

INTERACTION OF POLYENE ANTIBIOTICS WITH OTHER EUKARYOTIC SYSTEMS

PROTOZOA

The antiprotozoan properties of polyenes have received little attention but those studies have been made indicate that the mechanism of action is similar to the antifungal action. Indian workers [112,113,341–346] examined the effect of nystatin upon the protozoan *Leishmania donovani*, a human pathogen. Nystatin was rapidly absorbed by the organism and the presence of sterol in the medium antagonised polyene binding. Digitonin was found not only to inhibit nystatin binding but also released bound nystatin from the cell [344,346]. Nystatin was preferentially bound by those membrane fractions of *L. donovani* which contained the highest sterol concentrations [342]. Interaction of nystatin with the cell membrane caused the leakage of intracellular constituents from the cell, including 260 nm-absorbing material, phosphate, amino acids and small amounts of RNA and protein [343]. Nystatin inhibited endogenous and exogenous respiration of *L. donovani* [112]. The antibiotic induced cytological and cytochemical changes in the organism and subsequent lysis [341,345].

When the protozoan *Tetrahymena pyriformis* is grown in the presence of

ergosterol, the latter replaces the naturally occurring sterol tetrahymanol [347]. Ergosterol-grown organisms were twenty times more sensitive to polyenes than normal cells [348]. The interaction of filipin III with the membrane sterol of *T. pyriformis* has been monitored by UV, fluorescent and circular dichroism studies [155]. Filipin treatment produced 'pits', similar to those seen in fungal and *Acholeplasma* membranes and it was suggested that 'pit' formation occurred by a process of micelle-mediated fusion of the lipid layers of the membrane [155].

ERYTHROCYTES AND BLOOD COMPONENTS

Polyene antibiotics are known to be cytotoxic: intravenous application of polyenes into experimental animals may lead to haemolytic anemia, renal damage and subsequent death. Mammalian erythrocytes have been used to study polyene cytotoxicity. Polyene antibiotics induce rapid haemolysis of washed rat or human erythrocytes [109,117–119,349,350]. This is to be expected, since the membranes of erythrocytes are cholesterol-rich. Haemolysis requires the presence of an intact polyene molecule as saturation of the polyene conjugated double bond system, breaking the lactone ring by saponification or prolonged exposure to UV light destroyed the haemolytic properties of filipin [197]. Such derivatives were also unable to bind sterols or show any antifungal activity [193]. The ratio and extent of polyene-induced haemolysis was determined by the rate of antibiotic to erythrocyte membrane sterol rather than the antibiotic concentration [197]. Filipin promoted the most rapid lysis of erythrocytes. The affinity for sterol and the haemolytic ability of the four major components of the filipin complex were approximately equal [184]. The haemolytic ability of other polyenes was lower. Low concentrations (10 μg/ml) of filipin induced haemolysis of rat erythrocytes within 1 min. The same concentration of amphotericin B took 21 min before haemolysis while nystatin took up to 180 min [117]. Filipin produces drastic changes in erythrocyte membrane structure which can be seen as 'pits' in electron microscope preparations [233]. The haemolytic ability of individual polyenes corresponded approximately with their mammalian toxicity. In a series of polyene antibiotics, lipophilicity was found to decrease in the same order as erythrocyte haemolysis, i.e. etruscomycin > amphotericin B > nystatin > pimaricin [351]. The order of haemolytic potency amongst the major polyenes is filipin $\geqslant$ estruscomycin > amphotericin B > candidin > nystatin > pimaricin = candicidin.

Low concentrations of polyenes, other than filipin, produced more subtle effects on the erythrocyte membrane. Amphotericin B treatment elicited potassium fluxes from erythrocytes [119]. The influence of amphotericin B upon the permeability of mammalian erythrocytes has been studied by measuring move-

ments of nonelectrolytes, anions and cations [325]. Amphotericin B (0.5–2.0 μM) enhanced the rate of transfer of hydrophilic nonelectrolytes (glycerol and erythritol), anions (phosphate, lactate, chloride and thiocyanide) and cations (sodium and potassium). Nystatin also enhanced the permeability of all permeants but to a lesser extent [352]. Partial depletion of erythrocyte sterol had little effect upon permeability properties but reduced the effect of adding polyene.

Low concentrations of serum inhibit polyene haemolysis [109], so that direct interaction of antibiotic with erythrocytes *in vivo* may not be the cause of polyene-induced haemolytic anemia observed in patients [353,354]. The survival time of red blood cells *in vivo* was not reduced by amphotericin B and it was suggested that haemolytic anemia following polyene therapy was the result of suppressed erythrocyte production by bone marrow [353].

Polyenes have been shown to interact with other blood components. Amphotericin B and candicidin have been shown to combine with plasma proteins [355,356]. The aromatic hepatene candicidin bound to hypocholesteremic serum and to α and β lipoproteins or albumin in normal serum. The non-aromatic heptaene amphotericin B did not bind to hypocholesteremic serum and only bound to serum lipoproteins [355,356]. Nystatin has been shown to reduce the lysozyme activity of rat serum [357]. Amphotericin B and candicidin (levorin) inhibit the lecithin : cholesterol acyltransferase reaction in human plasma [358]. Filipin inhibits the extrinsic blood clotting system [359,360] and caused the aggregation of blood platelets [361]. Nystatin also caused platelet coagulation but to a lesser extent. Pimaricin and etruscomycin had no effect [361]. Polyene antibiotics (candicidin, nystatin and amphotericin B) increased the phagocytic response of experimental animals [362]. Nystatin and amphotericin B may co-operate with immune response mechanisms of the host when challenged with pathogens. Low levels of nystatin may penetrate into phagocytes and inhibit the mycellial transformation of engulfed *C. albicans* cells. Amphotericin B depressed capsule formation in *Crytococcus neoformans,* thereby enhancing the rate of phagocystic and intracellular killing of the pathogen [363]. Nystatin and amphotericin B exerted adjuvanticity on the primary *in vitro* antibody response of mouse spleen cells to heterologous erythrocyte antigens. In contrast, filipin suppressed the antibody response [364]. Polyenes were found to have no effect on lymphocyte proliferation [365].

MAMMALIAN TISSUE CULTURE CELLS

Many mammalian tissue culture cells are sufficiently resistant to polyenes to allow low concentrations of polyenes, usually amphotericin B or nystatin, to be included in tissue culture medium. Not all cells are resistant. It has been

demonstrated that certain cell lines (e.g. 3T3, 3T3-TK$^-$, K 455) were sensitive to amphotericin B, others were slightly more resistant (e.g. 3TP, BHK-B1), while some were resistant (e.g. 3T3-SV, BHK-T6, WG3) [120]. SV_{40} transformation of 3T3 cells resulted in increased polyene resistance [120].

It has been shown that nystatin caused swelling of Ehrlich tumour cells, and loss of 80% of cellular potassium [366] without affecting glycolysis or respiration, and this led to deformation of mitochondria, cytoplasmic changes and extensive vacuole formation. Mouse LS cells are ten to thirty times less sensitive to amphotericin B methyl ester, amphotericin or nystatin than are *C. albicans* cells. Sensitivity to perimycin and candicidin by the two cell types was similar, while mouse LS cells were more sensitive than *C. albicans* towards filipin [269]. Polyene antibiotics caused the release of potassium ions from mouse LS cells [269]. Cytological and cytochemical changes have been observed in chick embryo fibroblasts and mouse tumour cells after polyene treatment [121]. Nystatin and amphotericin B have been shown to be mitogenic in mouse spleen cells [368] and to inhibit the multiplication of chick embryo fibroblasts, Ehrlich tumour cells [121,369] and Novikov hepatoma cells [369]. Polyenes have also been shown to increase the rate of glycose utilization in mammalian fat cells [370] and increase the phospholipid turnover of beef thyroid slices [371].

Amphotericin B has been found to modify the membrane of the HeLa cells enhancing the uptake of foreign DNA [372] and cause the rupture of beef spermatozoa [373]. Low polyene concentrations do not damage cell lines [374], but it appears that higher concentrations damage the cell membrane, cause loss of metabolites and eventual death. There is, however, sufficient selectivity toxicity between tissue culture cells and fungi to allow the use of polyenes as antifungal agents in most tissue culture media.

MITOCHONDRIAL MEMBRANES

Studies with isolated mitochondria from rat liver of *Neurospora crassa* have shown that, even though polyene antibiotics are bound, they have little effect on oxidative phosphorylation, ion uptake, or electron transport [375]. The mitochondria of *Neurospora,* which contain ergosterol, remained viable after binding filipin [375]. Mitochondria have high phospholipid : sterol ratios in their membranes (approximately 40 : 1) and this may explain low polyene sensitivity.

CHEMICAL MODIFICATION OF POLYENE ANTIBIOTICS

The high mammalian toxicity and low aqueous solubility of polyene antibiotics has led to a search for derivatives with reduced toxicity, increased solubility and

increased stability, without reduction in antifungal activity. However, it appears that those features of the polyene molecule that contribute to antimicrobial activity are also responsible for cytotoxicity, instability and insolubility. The relationship between antifungal activity and cytotoxicity is not surprising if it is assumed that mammalian toxicity results from destruction of permeability properties of cell membranes. Any modification of the polyene molecule to decrease mammalian sterol–polyene interaction will also reduce yeast sterol–polyene interaction.

POLYENE FORMULATIONS WITH INCREASED WATER SOLUBILITY

The 'soluble' complexes of heptaenes commercially available are formulated with detergent-like substances, e.g. sodium desoxycholate or sodium lauryl sulphate, and do not form true solutions but consist of colloidal dispersions in an aqueous medium. The *in vitro* solubility of drugs with low aqueous solubility may be enhanced by coprecipitation with poly(vinyl pyrrolidone) (PVP) [376, 377]. The preparation of nystatin–PVP complexes has been described [378–380]. The solubility of PVP coprecipitates was eight to ten times greater than the parent compound [381] but the coprecipitates showed decreased antibiotic stability.

Various polyene–ion complexes have been developed. Water-soluble complexes of amphotericin B with calcium and oxalic or succinic acids have been described [382]. Stable water-soluble borate complexes of amphotericin B, candidin, candicidin, pimaricin have been developed [383]. An advantage of producing stable water-soluble iron(Fe(II) and Fe(III))–polyene complexes was that complexes retained the *in vitro* antifungal properties of the parent compound [384] while water-soluble calcium complexes showed enhanced antifungal action [385] and it has been suggested that amphotericin B–meglumine complexes were less toxic when used intravenously than desoxycholate complexes [386].

CHEMICAL MODIFICATION

The constrained nature of the macrocyclic lactone ring, the extensive unsaturation of the chromophore, and the variety of functional groups in the polyene molecule confers sensitivity to physical and chemical attack. Unfortunately, the resulting products are usually biologically inactive. Saturation of the chromophore or breaking the lactone ring by saponification reduced the affinity for sterol, the antifungal properties and the haemolytic activity [197].

The first attempts to reduce the toxicity of polyenes and increase water

solubility involved the reaction of amino groups in the molecule to give to corresponding *N*-acetyl derivatives [387,388]. These derivatives were water-soluble but showed reduced antifungal activity. *N*-Acetyl heptaenes showed antifungal activity, one-fifth to one-tenth of that of their parent compound [388]. Acetylated nystatin lost all its antimicrobial activity [203,387]. Aromatic heptaenes, e.g. candicidin, possess two amino groups (in the mycosamine and aromatic side-chain moieties). It is therefore possible to form monoacetyl (still amphoteric) and diacetyl (acidic) derivatives. The basic heptaene perimycin possesses no free carbonyl group and is rendered basic by its aromatic side chain. When acetylated, it forms an insoluble neutral derivative. Perimycin has been *N*-succinylated to produce an acidic derivative [387] which retained approximately 12% of the antifungal activity of perimycin.

A second approach to the production of water-soluble polyene derivatives was to modify the carbonyl group of amphoteric polyenes. Esterification of the free carboxyl group of amphotericin B or its *N*-acetylated derivative with diazomethane produced a methyl ester [389,390]. The hydrochlorides of the methyl esters of amphotericin B, nystatin and pimaricin were water-soluble, retained full activity of the respective parent but showed greatly decreased toxicity [91,391, 392]. Amphotericin methyl ester showed lower mammalian toxicity than did amphotericin B or amphoterin B–desoxycholate complex [393]. Longer chain alkyl esters of amphoteric polyenes were also antifungal and antitrichomonal but the lengthening of the aliphatic chain in the ester groups (methyl to butyl) produced a gradual decrease in antimicrobial action [394,395]. Alkyl esters of amphotericin B, nystatin and candicidin have been prepared, but although some derivatives were more active than the parent antibiotic, they were generally less active than the respective methyl ester [396,397].

Polyene antibiotics which possess a free amino group, e.g. amphotericin B, react with carbohydrates or their derivatives under appropriate conditions, yielding *N*-glycosyl derivatives. These derivatives formed water-soluble salts. The *N*-glucosyl and *N*-glucuronyl derivatives showed similar antifungal activity to the parent compound but reduced systemic toxicity [398,399] and proved effective when administered intraperitoneally in the treatment of experimental candidiasis in mice [400].

CLINICAL USES

Antifungal chemotherapy failed to achieve the early clinical and economic success of antibacterial antibiotics. In part, this was due to the relative unimportance of mycotic infections which, although often refractory, do not occur with great frequency in temperate climates. More important was the difficulty in

developing agents with sufficient selective toxicity to treat eukaryote pathogens within an eukaryotic host. Over the last twenty years the situation has slowly changed. As one bacterial infection after another became amenable to antibiotics, attention returned to antifungal compounds as the outstanding problem.

Human mycoses generally arise under conditions of metabolic stress or when defence mechanisms are impaired, e.g. during antibiotic, immunosuppressive or steroid therapy and in diabetic or leukemic patients. Several fungi, not normally thought of as pathogenic including *C. albicans* (a common commensal of mucous membranes) and *Aspergillus fumigatus* (a soil fungus with common ariel spores) can cause infection when the host is debilitated. This has led them to be termed the opportunistic fungi. Systemic mycoses developing from opportunistic fungi have caused post-operative deaths, notably in cases of transplant or open heart surgery. Opportunistic fungi may also develop fatal mycoses in children suffering from immune response defects and in cases of drug addiction. Candidial vaginitis is increasing in many countries due to increased promiscuity and the use of steroid-based contraceptives. Human mycoses pose a greater problem in under-developed tropical and sub-tropical countries and interest in the chemotherapy of tropical mycoses is increasing.

Polyene antibiotics show little or no antibacterial action but are potent antifungal agents. Polyenes inhibit many pathogenic and opportunistic fungi (*Blastomyces, Candida, Coccidiodes, Cryptococcus* and *Histoplasma* spp.), dermatophytes (*Epidermophyton, Microsporium* and *Trichophyton*) and moulds (*Aspergillus* sp.). Polyenes also inhibit pathogenic protozoans (*Entamoeba histolyca* and *Leishmania, Trichomonas,* and *Trypanosoma* spp.).

Generally, the antifungal activity within the polyene group increases with the number of conjugated double bonds. It has been reported that tetraenes and pentaenes have approximately one quarter of the anti-microbial activity of hexaenes and heptaenes against *C. albicans* [401]. Hamilton-Miller [6] determined the relative activities for a range of polyenes against *C. albicans* by calculating the minimum inhibitory concentration (MIC) in molar terms. This work demonstrated that the tetraene pimaricin exhibited an MIC of 7.5 μM, the pentaene filipin had an MIC of 7.4 μM, while those of the heptaenes, amphotericin B and candicidin, were 0.54 and 0.42 μM respectively. The tetraene nystatin was found to have an MIC of 0.33, much lower than other tetranenes, consistent with the hypothesis that nystatin is a 'degenerate' or modified heptaene. Heptaenes which contain an aromatic moiety, e.g. candicidin, are more active than those heptaenes without, e.g. amphotericin B [34,79,402]. *Table 3.6* shows the relative antifungal potencies of various polyenes.

Polyene antibiotics have slight surface-active properties and, although this property has been shown to play no role in the mechanism of action [103,345], the ability of polyenes to spread at surfaces makes them ideal drugs for topical

Table 3.6. RELATIVE ANTIFUNGAL ACTIVITY OF POLYENE ANTIBIOTICS *IN VITRO*

	Minimum inhibitory concentrations (μg/ml)					*References*
	Candida albicans	Cryptococcus neoformans	Histoplasma capsulatum	Trichophyton rubrum	Aspergillus niger	
Rimocidin	3–5	5–10	5	–	1.6	8
Pimaricin	3–6	3–10	1–1.6	3–15	1.8	18,354,404,405
Etruscomycin	2.5	–	25	–	–	406
Filipin	7.7	0.95	7.7	7.7	5	69
Nystatin	1–3	1.6	1.6	6–14	2	407–411
Amphotericin B	0.2–4	0.6	0.04–1	30	0.09	13,404,407,412–414
Candicidin	0.04	0.16 [a]	2.5 [a]	1.28 [a]	0.8	415
Perimycin	0.06	0.5	–	–	0.03	413,416

[a] Author's unpublished observations.

Table 3.7. TOXICITY OF POLYENE ANTIBIOTICS IN SMALL MAMMALS

	LD_{50} *(mg/kg)*					
	iv	*ip*	*sub-c*	*Oral*	*Animal*	*Reference*
Amphotericin B	4–6.6	280–1640		>8000	Mice	6,404,418
Candicidin		2.1–7	160–280	98–400	Mice	6,404
Nystatin	3	45	2.4	8000	Mice	6,404
Pimaricin	5–10	250	5000	1500	Rats	6,18

iv, intravenous; ip, intraperitoneal; sub-c, subcutaneous.

application. Due to their high parenteral toxicity (*Table 3.6*) the use of most polyenes has been restricted to the treatment of topical or gastro-intestinal infection. Orally administered polyenes are poorly absorbed [145,403], if at all, and hence exhibit low toxicity when administered by this route (*Table 3.7*). Polyenes are best tolerated orally, followed by the subcutaneous and then intraperitoneal routes.

Chemical modification may considerably reduce the toxicity of polyenes. Amphotericin B methyl, ethyl, propyl or butyl esters have been shown to have LD_{50} values ten times greater than that of the parent compound when intravenously injected into mice [396]. Similarly, the methyl to butyl esters of candicidin exhibited LD_{50} values ten to forty times greater than that of candicidin when injected by the intraperitoneal route into mice [396].

Filipin is far too toxic to mammalian cells to be used in antifungal therapy. Many of the polyene antibiotics have been used in experimental and clinical mycotic infections in man and animals but only four polyenes are available commercially in the United Kingdom: amphotericin B (Fungizone, Fungilin), candicidin (Candeptin), nystatin (Nystan, Nitacin) and pimaricin (Pimafucin, Nantamycin). These antibiotics have been used in the treatment of most mycoses and as they combine antifungal action with activity against *Trichomonas* sp. [419] polyenes are attractive for the treatment of vaginitis.

ANTIBIOTIC USES OF FOUR COMMERCIALLY AVAILABLE POLYENES

Nystatin

The therapeutic use of nystatin has been reviewed [409,420]. This antibiotic has been used successfully in the treatment of monilial infections of the skin, nails and mucosal surfaces. Nystatin given orally rapidly clears candidosis of the

alimentary tract. As nystatin is poorly absorbed from the gut, oral nystatin therapy is ineffective in the treatment of systemic mycoses. However, oral application may ameliorate systemic mycoses by reducing infection in the gut. The high parenteral toxicity of nystatin excludes its use by this route for the treatment of systemic mycoses. Oral nystatin is used as a therapeutic adjunct to prevent or minimize fungal proliferation during antibacterial or immunosuppressive therapy and to remove foci of fungal infection in the gut before transplant surgery. Cases of allergic contact dermatitis resulting from nystatin have been reported [421–423].

Amphotericin B

Amphotericin B has been the mainstay of the therapy of acute systemic fungal infections (including histoplasmosis, blastomycoses, candidosis, coccidioidomycosis and cryptococcal meningitis [107,424] for twenty years. This antibiotic may be given orally or more usually intravenously by infusing the antibiotic over several hours. As intravenous amphotericin B may have side effects, a maximum total dosage of 3 g of intravenous amphotericin B has been recommended [425]. Larger doses, up to 16 g, can be tolerated orally. Although orally given amphotericin B only gives rise to very low blood levels, oral therapy has been reported effective in the treatment of blastomycosis, and coccidioidomycosis [426] but generally parenteral treatment of amphotericin B is preferred for systemic mycoses. In man infusion of amphotericin B (usually as the deoxycholate complex) is often associated with nausea, anemia, fever and kidney damage. It is often stated that intravenous amphotericin B therapy should only be given in cases of extremis [427]. Although the toxic side effects of systematically given amphotericin B must not be underestimated, Symmers [428] commented upon the 'pharmacophobia' which often accompanies the use of this antibiotic and suggested that amphotericin B therapy is often withheld, to the detriment of the patient, until the symptoms of systemic mycosis become so severe that subsequent polyene therapy has little effect. He cited six fatal cases of mycotic infections (including cryptococcal meningitis, histoplasmosis and blastomycosis) which he believed could have been prevented if amphotericin B therapy had been initiated earlier in the development of the disease.

Prolonged intravenous amphotericin B therapy has been shown to produce haemolytic anemia and renal damage. Although polyene antibiotics are known to lyse erythrocytes *in vitro*, it is not thought that *in vivo* lysis caused the observed anemia (see p. 150) but resulted from inhibition of bone marrow erythrocyte synthesis [353].

Prolonged therapy with amphotericin B may lead to kidney damage, which may be irreversible [429]. Nephrotoxicity and hypokalemia are the major

factors limiting amphotericin B therapy [430–436]. It may be that the hypokalemia [432–435], renal sodium wasting [435] and gradient-limited renal tubular acidosis [436] observed in the course of systemic amphotericin B therapy may be due to the effect of the antibiotic on the distal nephron. Amphotericin B was shown to induce changes in kidney structure including swelling, hyperplasia especially of the glomeruli and interstitium. The swollen tubes were found to contain calcium deposits, formed as a result of amphotericin B-induced renal acidosis [437,438]. Other studies have indicated that glomerular lesions were common in amphotericin B-treated patients but the damage was reversible when the total dose was less than 5 g [436]. The danger of renal damage was greatest in those patients with a history of renal impairment.

Renal tubular acidosis can occur during amphotericin B therapy [439,440]. Renal tubular acidosis and hykalemia can be easily corrected with oral potassium therapy [436,439,441]. Hydrocortisone and heparin are sometimes used in conjunction with polyene therapy to reduce toxic side effects [442]. The immediate reactions to intravenous amphotericin B therapy (nausea, vomiting and fever) can be controlled to some extent by usually antihistamines and hydrocortisone [443,444] but reports that some of the symptoms of nephrotoxicity may be overcome by mannitol supplementation [445] have been disputed [446].

Any selectively in the action of polyene antiobiotics in eukaryotes must be based on the differing affinities of polyenes for individual sterols. The use of amphotericin B as a drug in the treatment of mycotic infections in man is indicative of the apparently greater affinity of the antibiotic for the fungal sterol ergosterol rather than for the mammalian sterol cholesterol.

Amphotericin B is clinically effective in many systemic fungal diseases including blastomycosis [447–450], coccidioidomycosis [447,451,452], cryptococcosis [447,453,454] cryptococcal meningitis [455], histoplasmosis [447,456–459] *Candida* meningitis [460] *Candida* endocarditis [461], *Sporotrichium* meningitis [462], mucormycosis [447,463,464] and aspergillosis [465–467]. Amphotericin B has also been used in the treatment of amoebic meningo-encephalitis [468,469], leishmaniasis [470,471] and vaginal trichomoniasis [472].

Amphotericin B is also used as a topical agent in the treatment of mycoses but to a lesser extent than is nystatin [409]. Amphotericin B has been used successfully to treat epithelial keratitis due to *Rhodotorula* sp. [473] and fungal endopthalmitis [474].

Candicidin

Candicidin is used for the treatment of localised infections topically applied to the vagina, skin or oropharyngeal regions. Candicidin inhibits a wide range of

fungi at lower concentrations than other polyenes [34,475–477]. Candicidin has been used extensively for successful treatment of vaginitis caused by *C. albicans* [475–486], for the treatment of intertriginous and paronychial moniliasis [487,488], and in the treatment of visceral leishmaniasis [489].

Pimaricin

The properties and therapeutic uses of pimaricin have been extensively reviewed by Raab [490]. Pimaricin has found topical application in the treatment of candidial vaginitis [491–496], trichomonal vaginitis [496–500], non-specific vaginitis [501], keratitis and other eye infections [502–505] and cutaneous infections due to *Candida* and *Aspergillus* spp. [506].

SYNERGISTIC INTERACTION WITH POLYENE ANTIBIOTICS

Attempts have been made to reduce the systemic toxicity of polyenes by replacing part of the polyene dose with alternative antimicrobial agents. These *in vitro* experiments show some promise for the chemotherapy of systemic mycoses.

2-Deoxyglucose interferes competitively with glucose metabolism of yeasts [507]. *In vitro* synergistic antifungal activity has been reported between this compound and amphotericin B [508]. Medoff, Comfort and Kobayashi [509] have reported a synergistic effect between 5-fluorocytosine (5-FC) and amphotericin B against yeasts. The existence of this synergy has been disputed [510, 511] and this has led to the suggestion that synergy only occurs in certain isolated of pathogenic yeasts [512–516]. Synergy has been reported between 5-FC and candicidin [517]. Rifamycin has been shown to inhibit bacterial and animal nucleic acid polymerases [518]. Synergism between amphotericin B and rifamycin, or its semi-synthetic derivatives have been reported for a wide range of opportunistic and pathogenic fungi [519–523].

CHEMOTHERAPY OF BENIGN PROSTATIC GLANDULAR HYPERPLASIA

Gordon and Schaffner, when determining the oral toxicity of candicidin, used relatively old dogs. On feeding the antibiotic (20 mg/kg body wt.) for thirty days, there was a dramatic reduction in prostate size accompanied by reduced serum cholesterol levels [524]. Candicidin therapy produced a 30–60% reduction in gland size with marked histological changes involving diminution of gland diameters and epithelial cell heights associated with reduced congestion, granulation and papillations. These effects were observed to persist for several months

after withdrawal of treatment. Those polyenes exhibiting least antifungal activity were found to have little effects on hypertrophied prostates. The heptaenes, amphotericin B and candicidin, two of the most antifungal polyenes, had the greatest effect on the prostate of rats [525] and dogs [145]. Candicidin and pimaricin were shown to inhibit prostate gland development in young rats but equivalent concentration of amphotericin B or nystatin had little effect [526].

In elderly males, benign hypertrophy of the prostate is of great clinical importance. It results in urinary obstruction, secondary changes in the urinary tract due to back pressure and risks of secondary infection. Chemotherapy of this condition is attractive as surgery is not always practicable in elderly patients.

Successful human trials [145,527–532] have demonstrated that candicidin can reduce prostatic glandular hyperplasia. Prolonged (3–12 months) oral therapy caused a rapid improvement, reduction in glandular enlargement, lessening of symptoms of urinary retention, hesistancy and nocturia. Oral therapy for up to two years revealed no toxicity or adverse reactions. Prostate hyperplasia may result from prolonged androgen stimulation and the reduction in prostatic size may be a consequence of lowered levels of androgen precursors, e.g. cholesterol. It has been shown that amphotericin B reduced the conversion of testosterone to dihydrotestosterone in the prostate [533] and steroid production in adrenal cells [534].

ANTIHYPERCHOLESTEROLEMIC PROPERTIES OF POLYENES

Hypercholesterolemia, raised blood cholesterol levels, is a common manifestation of disordered body function arising from diverse physiological and pathological processes, including genetic, dietary and endocrine disorders [587]. Investigation of the effects of candicidin upon benign prostatic hyperplasia suggested that polyenes, particularly the heptaenes, could have potential as antihypercholesterolemic agents. Oral administration to dogs for three weeks at 5–40 mg/kg produced reduced sterol levels. Since little, if any, candicidin is absorbed from the gut, it was suggested that the antibiotic combines with dietary sterol and that in the extra-hepatic circulation, these complexes cannot be absorbed and are excreted in the faeces [535]. This was confirmed when it was shown that small concentrations of certain polyenes, particularly the aromatic heptaenes, reduced the plasma sterol levels of chicks [536,537] and promoted excretion from the digestive tract. Similarly, candicidin [145, 538–540], amphotericin B- [540,541] or hamycin-treated [542,543] rats showed dose-related decreases in plasma cholesterol levels, with increased sterol and total lipid levels in the faeces. When treatment ceased, normal sterol levels were rapidly restored [145]. Intravenous injection of amphotericin B into rats rapidly reduced serum cholesterol levels [544,545].

Significant reduction in serum cholesterol levels can also be demonstrated in animals simultaneously fed polyenes and high sterol diets. Dogs appeared more able to maintain their serum cholesterol levels when fed sterols and filipin, amphotericin B or candicidin [145,535] but nystatin had no effect. The anti-hypercholesterolemic effect was not simply due to the prevention of sterol uptake [538,539]. A consequence of the excretion of sterol–polyene complexes in the faeces is the loss of bile salts (which are steroids) and this tends to remove sterol from the body. This resulted in increased hepatic synthesis, increased utilization of adipose fat and reduced sebum levels. Candicidin has been suggested as an antihypercholesterolemic agent in man [546].

It has also been reported that oral candicidin therapy of chickens resulted in a lower incidence of coronary artery lesions and reduced severity and incidence of aortic atheroschloris regardless of whether or not the birds received additional dietary sterol [547]. Amphotericin B therapy has been shown to reduce the cortisterone blood levels in experimental animals [548,549]. Oral heptaene therapy (especially with candicidin) has been used in the treatment of acne in males without producing any hyperaoestrogenic effects [550].

OTHER USES

Polyene antibiotics are used extensively to control fungal contamination, mainly yeasts and moulds, in microbiological medium, in biological samples from which it is hoped to isolate bacteria, and especially in cell and tissue culture media [551–559]. Polyenes are used in conjunction with benzyl penicillin and streptomycin to suppress microbial contaminants in tissue cultures.

Polyenes have been used to stimulate and increase the cardiac output of experimental animals [560–567].

Nystatin has been incorporated into dentifrice for treatment of periodonta [568] and used in conjunction with rifamycin and corticosteroids for the treatment of hair loss [569].

ACTIVITY

Nystatin is the only polyene antibiotic whose activity is quoted in international units (pure nystatin contains over 5500 U/mg and the international standard for nystatin contains 3000 U/mg [570]). International standards have been proposed for amphotericin B [571].

Recommended procedures have been published for the microbiological assay of most polyenes [572–574] and for the assay of polyenes from serum and animal tissues [575,576].

CONCLUSIONS AND FUTURE PROSPECTS

The antimicrobial action of polyenes depends upon hydrophobic interaction between the antibiotic and membrane-located sterols. The use of polyene antibiotics in topical and especially systemic antifungal therapy depends upon the greater binding affinity between the polyene and fungal ergosterol than that between the antibiotic and mammalian cholesterol. Filipin and amphotericin illustrate two extremes of polyene action.

Filipin exhibits its greatest potency against erythrocytes, while amphotericin is most effective against fungi. The haemolytic and antifungal effects of filipin are more effectively blocked by cholesterol, whereas the equivalent properties of amphotericin B are more effectively prevented by ergosterol [144,577]. Fluorimetric studies have indicated that filipin exhibited a preference for cholesterol over ergosterol [150]. No such preference was demonstrated for amphotericin B [159]. Filipin shows greater cytotoxic and haemolytic properties than does amphotericin B because of its greater affinity for cholesterol rather than ergosterol. Similarly, the greater antifungal activity of amphotericin B reflects its greater avidity for ergosterol over cholesterol [118,271,578]. Studies of the polyene susceptibility of model and natural membranes [244,245,579] have suggested that amphotericin B demonstrates greater affinity for ergosterol-containing membranes, while filipin exhibited its greater affinity for cholesterol-containing membranes.

The antibiotic properties of the remaining polyenes fall somewhere between filipin and amphotericin B in their relative affinities for cholesterol and ergosterol. If the precise nature of the interaction between polyenes and sterol can be elucidated, then it may prove possible to find those structural features of the polyene molecules which account for sterol specificity. This would allow the synthesis of polyene or polyene-like antibiotics with increased affinity towards ergosterol and/or reduced cholesterol affinity. Such agents could be safely used for systemic antifungal therapy. Until this is possible, antifungal therapy is restricted to polyene antibiotics discovered as a result of antibiotic screening or their chemical derivatives. Although much progress has been made, we are some way from the goal. The syntheses of polyene methyl esters, particularly amphotericin B methyl ester [389,390], was a significant advance, producing derivatives which were water-soluble with reduced nephrotoxicity and equivalent systemic antifungal activity [391–400,580–586]. Amphotericin B methyl ester was not de-esterified in the body [585]. Esterification either increases the affinity of the antibiotic for ergosterol or decreases the affinity for cholesterol (or both). Future chemical modification may produce other derivatives with an absolute requirement for ergosterol. Polyenes have been shown to reduce serum sterol levels [535–545] and prostatic hyperplasia [524–533] and it has been

suggested that they could be used clinically in the treatment of atherosclerosis [547] and acne [550]. If it proves possible to produce sterol-specific polyenes, then the use of this group of antibiotics in a whole range of steroid-based disorders becomes a distinct possibility.

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4 Functional Modifications and Nuclear Analogues of β-Lactam Antibiotics – Part II*

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* Part I: Chapter 8, Vol. 12 of this Series.

INTRODUCTION

The different penicillins and cephalosporins are very widely and intensively used, powerful agents in human chemotherapy, without toxic properties. Following the discovery of the natural penicillins and cephalosporins, the main aim of chemists was to elucidate their structures, but from the early sixties on the investigations concentrated more and more on their chemical transformations. Among the culminations of this huge work were 'The Chemistry of Penicillin' (1949) [1] and 'Cephalosporins and Penicillins, Chemistry and Biology' (1972) [2], which are very relevant books of reference with regard to many questions. The chemical transformations and synthetic chemical work exhibit many facets. The isolation of 6-APA and 7-ACA served as the source of new semi-synthetic derivatives with high and broad activities, while intensive investigations of the structural transformations of natural penicillins – easily and relatively cheaply available by fermentation – are also progressing rapidly.

However, these are not the sole possibilities, because the total synthetic trends are also of great interest; to date numerous compounds have been produced, thereby helping towards a better understanding of structure–activity relationships and providing new antibacterials and compounds of other uses.

This chapter summarizes the recent developments in the field of the synthesis of nuclear analogues of penicillins, cephalosporins, monocyclic β-lactams and other penicillin and cephalosporin analogues. An account is given of the available data on other β-lactams of biological interect and certain attempts to synthesize some of these molecules with the help of our recent knowledge on biosynthesis.

A review dealing with such a rapidly-developing field as the β-lactams is by no means able to cover all the literature data available from the different sources. The authors therefore intended the treatment of the material to be more critical than exhaustive.

ABBREVIATIONS

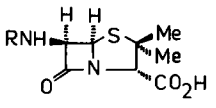
Standard abbreviations are used throughout this chapter, in accordance with those used in Part I of this work. Abbreviations for reagents, protecting groups, etc. are mostly in accordance with those used by Fieser and Fieser [3].

R at position 6 or 7 (e.g. RNH–) means an acyl group unless otherwise stated. G, V, A, T, M, O, ClO at position 6 or 7 (e.g. VNH–, ANH–, etc.) correspond to the side-chains of benzyl- and phenoxymethylpenicillin, ampicillin, cephalothin, methicillin, oxacillin and cloxacillin, respectively. In addition, X at position 6 or 7 is a substituent other than an acyl substituent, e.g. halogen, methoxy, etc.

ClAT	Chloramine T
DCC	Dicyclohexylcarbodiimide
DHP	Dihydropyran
DME	Dimethoxyethane (glyme)
DPP	Dipyridine phosphate
HMPA	Hexamethylphosphoric triamide
LTA	Lead tetra-acetate
MCPA	*m*-Chloroperbenzoic acid
NBS	*N*-Bromosuccinimide
Pht	Phthaloyl
PNB	*p*-Nitrobenzyl
pTsOH	*p*-Toluenesulphonic acid
TEA	Triethylamine
TFA	Trifuloroacetic acid
TFPA	Trifluoroperacetic acid
Troc	β,β,β-Trichloroethoxycarbonyl

STRUCTURAL CONSIDERATIONS

Penicillins (1) and cephalosporins (2) of either natural or semi-, partly or totally synthetic origin have in most cases two fundamental units, the penam (3) [4] and cepham (4) [5] systems.



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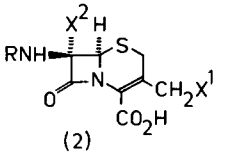

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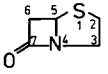

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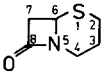
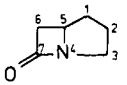


(4)

The names penam and cepham or cephem are widely used and are applicable to most of the natural or semi-synthetic derivatives [6] without difficulties. In the case of nuclear analogues, however, this nomenclature causes various problems. In order to solve this question, Bose [7] has suggested a nomenclature for fused β-lactams. Keeping the 'unofficial' numbering, he gave the generic names heptam (5), octam (6), nonam (7) and decam (8) to these bicyclic systems.



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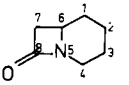

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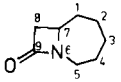

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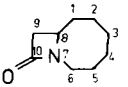

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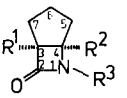
For bicyclic β-lactams of other types he suggests the names neoheptam (9), neooctam (10), etc. In this 'neo'-series the suggested numbering system is based



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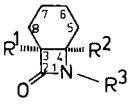


(10)

on the numbering of the monocyclic β-lactam compounds.

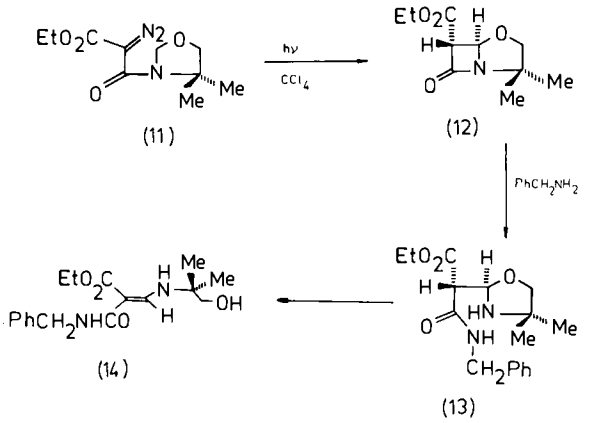
OXA-ANALOGUES OF PENICILLINS AND CEPHALOSPORINS

As a result of intensive research in the field of β-lactam compounds, partial and total syntheses have been developed for sulphur-free fused β-lactam systems, and such analogues of natural origin have also been isolated. These compounds provided useful data concerning the structural requirements for β-lactamase inhibitors and antibacterial substances of this type.

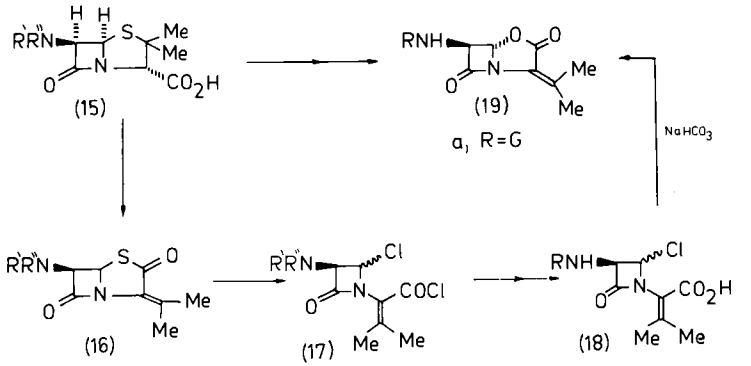
PENICILLIN ANALOGUES: OXAHEPTAMS

Oxa-analogues of penicillins are of current interest because of the possible role of the sulphur in penicillins during the interaction with enzymes involved in the

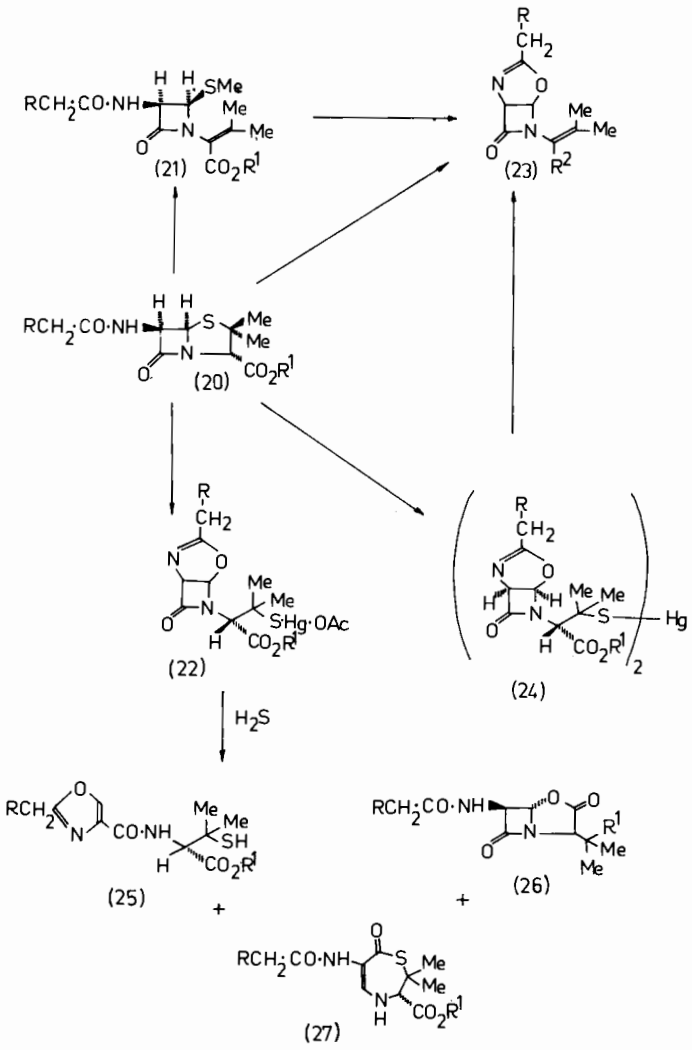
cross-linking process of bacterial cell wall synthesis [8–10]. From this point of view the method using photolytic ring-closure, developed by Lowe [11], is an interesting possibility. This method is based on the observation of Corey and



Felix [12]. It has been employed by Golding and Hall [13] for the synthesis of a 1-oxaheptam derivative (12). Although spectroscopic evidence clearly indicated the formation of compound (12), different attempts failed to yield it in pure form. The presence of the β-lactam C=O absorption at approximately 1790 cm^{-1} is a sign of chemical and – in most cases – biological reactivity [14]. In fact, (13) is formed rapidly following the addition of benzylamine to the reaction mixture containing (12).

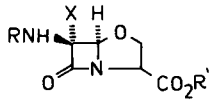


The formation of another 1-oxaheptam derivative is also of some interest. This compound (19) is obtained in 40–48% yield from a chloro-azetidinone derivative (17) [15–17].



A similar compound (26) was prepared by Stoodley and Watson [18], using a different reaction sequence. Although compound (26) and the mercury-free mercapto derivative of (22) are mentioned as compounds without significant antimicrobial activity, it is noteworthy that the former has a minimal inhibitory concentration of 10 μg/ml against the most sensitive strains ((26a), R = Ph, R = SH) [19].

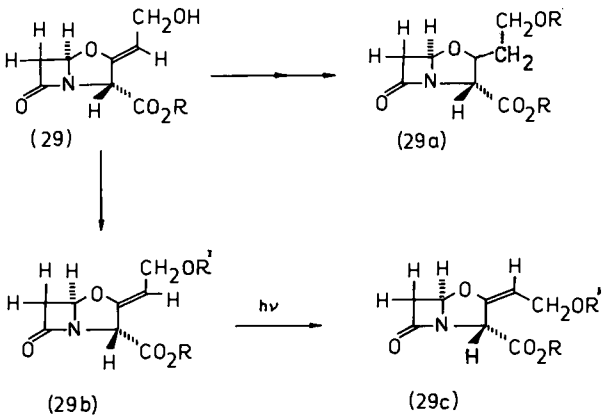
Compounds of type (28) are further examples of 1-oxaheptam penicillin



(28)

analogues prepared by a total-synthetic procedure [20].

During a research programme aimed at the isolation of natural products capable of inhibiting β-lactamase, a new 1-oxaheptam derivative (29) was isolated from a culture of *Streptomyces clavuligerus* [21]. This compound, clavulanic acid ((29), R = H), as its sodium salt (R = Na) proved to be a potent irreversible inhibitor of different β-lactamases [22]. In the presence of 5 μg/ml

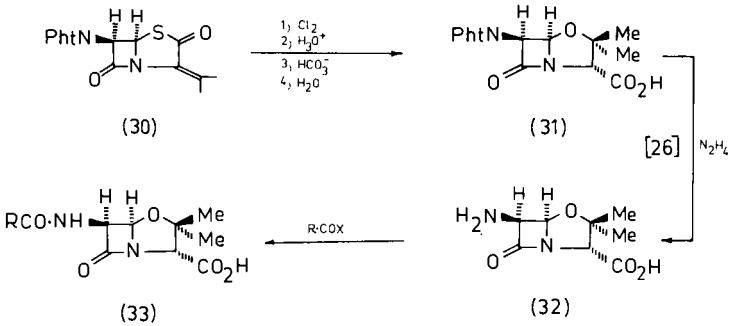


(29) (29a)

(29b) (29c)

of this sodium salt the *in vitro* minimum inhibitory concentration of ampicillin was reduced from >1000 μg/ml to <1.0 μmg/ml against a β-lactamase-producing resistant *Staphylococcus aureus* strain. Other derivatives of clavulanic acid (29) are also of interest. The saturated *O*-acyl derivative (29a) [22a] is claimed to be antibacterial while the unsaturated *O*-acyl compound (29b) is a non-toxic β-lactamase inhibitor [22b]. Compound (29b), (R = V, R′ = CH_2Ph) shows inhibitory activity against different β-lactamases: ID_{50} against β-lactamase from *S. aureus* Russell (7.5 μg/ml), *E. coli* JT4 (2.5 μg/ml) and *E. coli* JT10 (1.5 μg/ml). Photoisomerized product of type (29c) [22c] is also mentioned as β-lactamase inhibitor and antibacterial agent. It is suggested that it be used together with penicillins and cephalosporins to treat a wide range of infectious diseases.

Preparation of the 1-oxaheptam derivative, which is a true analogue of penicillins, was achieved by Wolfe. In his reaction sequence, the ring opening of penicillins and cephalosporins as described by Kukolja [23–25] is used. In the case of a phthalimido (PhtN) substituent, the 6-APA analogue (32) is formed in the reactions outlined [16].



Starting with a natural penicillin (34), the 6-aminoanhydropenicillanic acid ((35), R = R′ = H) is formed, and the *p*-toluenesulphonic acid salt (RR′NH = TsOH · H_2N– in (35)) is transformed to the 1-oxaheptam analogue of penicillins (33) [16].

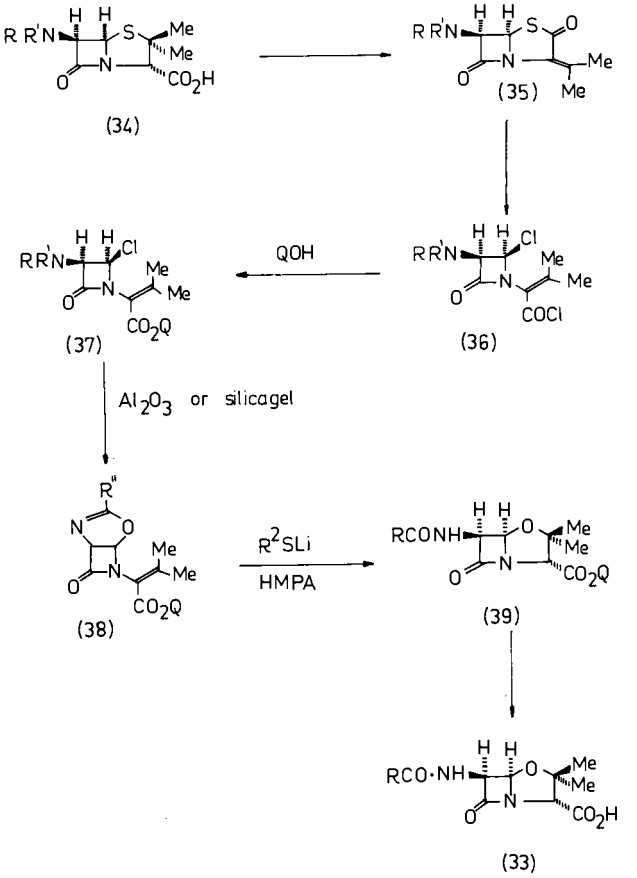


Table 4.1. ANTIBACTERIAL SPECTRUM OF OXAPENICILLIN G ((33), R = $PhCH_2$) [16]

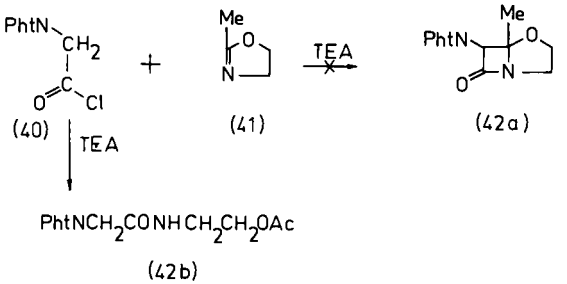
Strains	*MIC (μg/ml)*	
	Penicillin G	*Oxapenicillin G*
D. pneumoniae +5% serum [a]	0.004	8
Str. pyogenes + 5% serum [a]	0.004	8
S. aureus Smith (at 10^{-4} dil.)	0.016	16
+ 50% serum	0.06	63
S. aureus BX 1633-2 (at 10^{-3} dil.)	125	63
S. aureus BX 1633-2 (at 10^{-2} dil.)	>125	125
S. aureus methicillin res. (at 10^{-3} dil.)	125	32
Sal. enteriditis (at 10^{-4} dil.)	0.13	63
E. coli Juhl (at 10^{-4} dil.)	32	63
K. pneumoniae (at 10^{-4} dil.)	2	63
Pr. mirabilis (at 10^{-4} dil.)	1	63
Pr. morganii (at 10^{-4} dil.)	>125	63
Ps. aeruginosa (at 10^{-4} dil.)	>125	63
Ser. marcescens (at 10^{-4} dil.)	>125	63

[a] 45% AAB at 10^{-3} dil. + 50% medium.

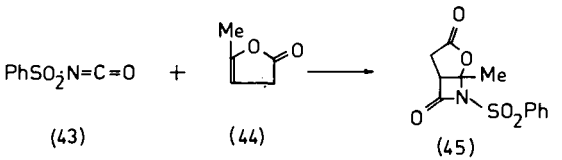
The antibacterial spectrum of the 1-oxaheptam 'oxapenicillin G' analogue of penicillin G is outlined in *Table 4.1.*

It is an interesting characteristic of this compound that it has a MIC range of 8–125 μg/ml against different organisms. These values are more constant than those for penicillin G.

Starting with 1-oxa-2,2-dimethyl-6β-aminoheptam-3-carboxylic acid (O-6-APA) (32), the whole range of side-chains of clinical penicillins have been employed in the 1-oxaheptam series [16].



In an attempted cycloaddition formation of 1-oxaheptam, (42a) was expected [27]. The product, however, proved to be an open-chain compound, 2-

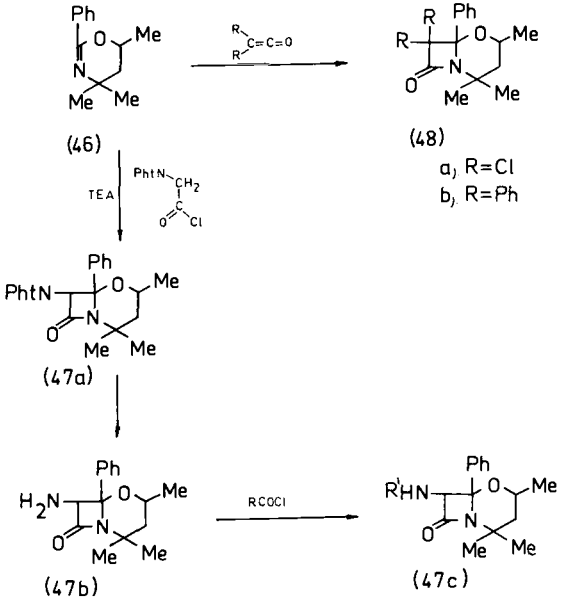


(43) (44) (45)

phthalimidoacetamidoethyl acetate (42b) [28]. Another cycloaddition reaction with a similar compound (44) and benzenesulphonyl isocyanate resulted in the formation of a 5-oxaneoheptam (45) [29].

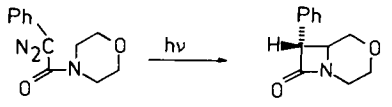
CEPHALOSPORIN ANALOGUES: OXAOCTAMS

1-Oxaoctam derivatives are oxygen analogues of cephalosporins. Sheehan and Dadic employed phthalimidoacetyl chloride for the preparation of 1-oxaoctam (47a) [30]. Hydrazinolysis gave 2,4,4-trimethyl-6-phenyl-7-amino-1-oxaoctam (47b), an analogue of 7-aminocephalosporanic acid. Cycloaddition of dichloro or



diphenyl ketene to the same starting material (46) resulted in the formation of similar oxaoctams, (48a) and (48b), respectively [31].

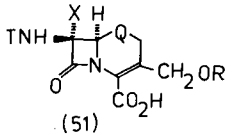
Another distant nuclear analogue of cephalosporins is compound (50), a 2-oxaoctam derivative, 2-oxa-7α-phenyl-octam, formed by photolysis of the corresponding diazoamide [32].



(49) (50)

In the light of the results of structure–activity relationship studies carried out on β-lactam antibiotics, however, no antibiotic activity can be expected in the case of nuclear analogues as distant as (48) or (50) [33–35].

These first attempts were very soon followed by total and partial syntheses of true nuclear oxa-analogues of cephalosporins. The Merck group reported the total synthesis of the 1-oxaoctam analogue (51b) of cephalothin (51a): (±)-1-



(51)

a) X = H, Q = S, R = Ac
b) X = H, Q = O, R = Ac
c) X = OMe, Q = S, R = $CO{\cdot}NH_2$
d) X = H, Q = S, R = Ac

oxacephalothin. They have developed this interesting total synthesis and already employed it successfully for the total synthesis of (±)-cephalothin (51d) [36,37], (±)-Cefoxitin (51c) [38] and other analogues [39–41]. By the synthesis of the 1-oxaoctams mentioned above, it was also intended to study the possible role played by the sulphur in cephalosporins during the interaction with

Table 4.2. COMPARISON OF MINIMUM INHIBITORY CONCENTRATIONS (MIC, μg/ml) OF CEPHALOTHIN (51a) AND ANALOGUE (51b) [42]

Strain	*Sodium salt of*	
	(51a)	*(51b)*
S. aureus 2865	>0.39	<0.39
Str. pyrogenes 3124	<0.39	<0.39
Klebsiella sp. 2882	6.25	3.12
E. coli 2884	6.25	6.25
Shigella sp. 2880	6.25	3.12
Salmonella schottmuelleri 2837	6.25	3.12

the target enzymes of the bacterial cell wall. It was pointed out, however, that the smaller oxygen atom in this oxa-analogue (51b) could result in a more strained bicyclic ring system, thereby increasing the reactivity and bioactivity.

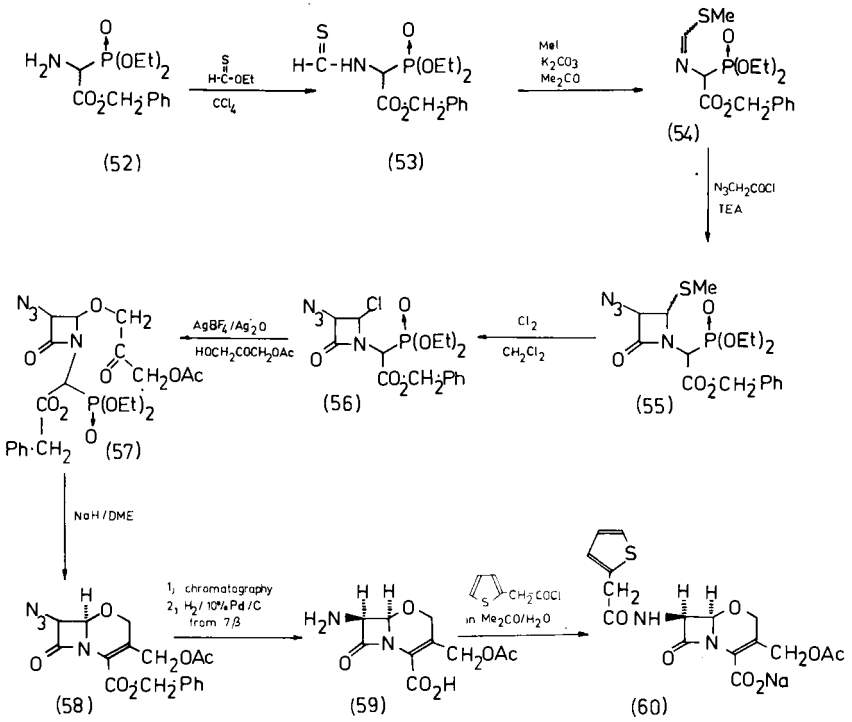
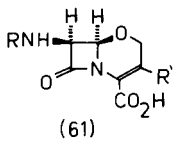
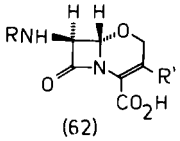


Table 4.2 contains the available bioactivity data for (±)-1-oxa-3-acetoxymethyl-7β-thienylacetamido-hept-3-em-4-carboxylic acid (51b).

The anhydro-6-phthalimidopenicillin method [16] was successfully employed for the synthesis of oxaoctams (61) and (62) [43]. These compounds are also

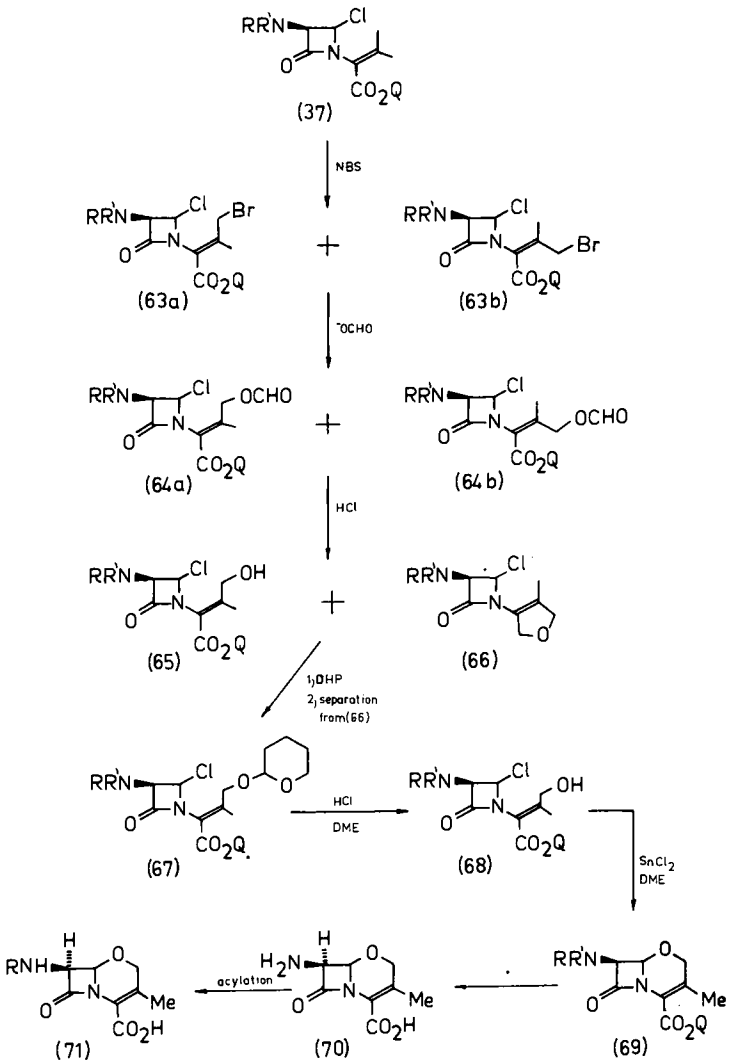


(61)

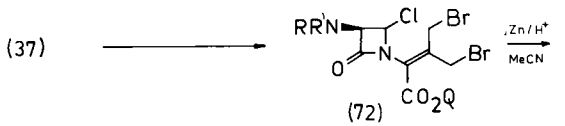


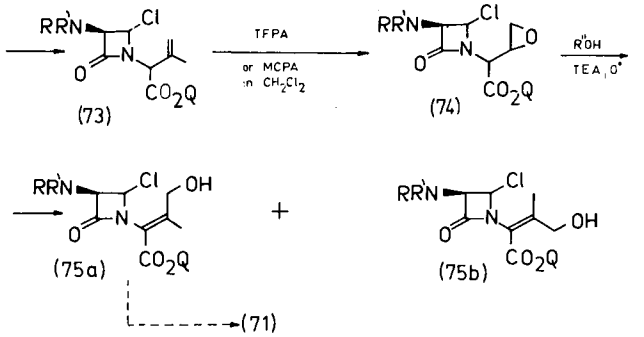
(62)

claimed to be active against a wide range of micro-organisms [43], similarly to (60). Starting from anhydropenicillin, the desired end-product was obtained via the intermediates (37) and (65) [43–45]. Another possibility for the preparation of intermediate (65) is through the epoxide (74). The 3-hydroxy-1-oxa-

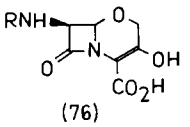


octam derivative ((76), 3-hydroxy-7β-acylamino-1-oxaoct-3-em-4-carboxylic acid) is the result of a similar reaction sequence [43]. The corresponding anti-





bacterial 2-oxaoctam derivatives have also been obtained by a totally synthetic route [43a].

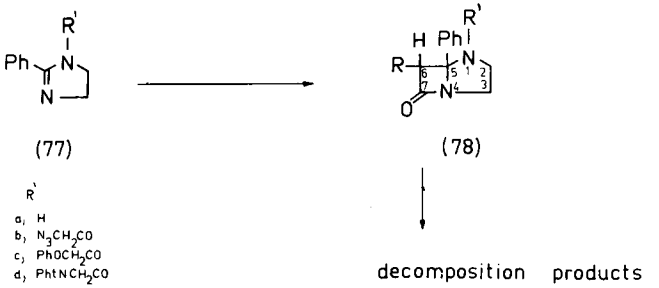


AZA-ANALOGUES OF PENICILLINS AND CEPHALOSPORINS

The rapid development in the field of β-lactam antibiotics has led to the synthesis of nuclear analogues containing nitrogen instead of sulphur. These compounds are potential antibacterial agents and may also help towards a better understanding of the structural requirements with special regard to the building elements of the ring systems.

PENICILLIN ANALOGUES: AZAHEPTAMS

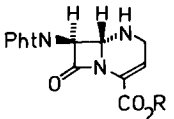
Azaheptams of type (78) are unstable distant analogues of penicillins. These compounds have been obtained in crude form [46], but purification attempts



lead only to decomposed products. Nevertheless, the lack of a carboxyl group in position 3, and the presence of a bulky substituent in position 5, suggest that compounds of this type would be inactive as regards to antibacterial activity.

CEPHALOSPORIN ANALOGUES: AZAOCTAMS

Azaoctams can be obtained and purified more easily then the corresponding azaheptam derivatives. A very close analogue of cephalosporins is the phthalimido derivative (79), which was obtained by partial synthesis from anhydropenicillin (Wolfe method) [15]. In the light of the stereochemical requirements for bio-



(79)

activity of bicyclic β-lactams, however, no antibacterial activity can be expected in this case [47].

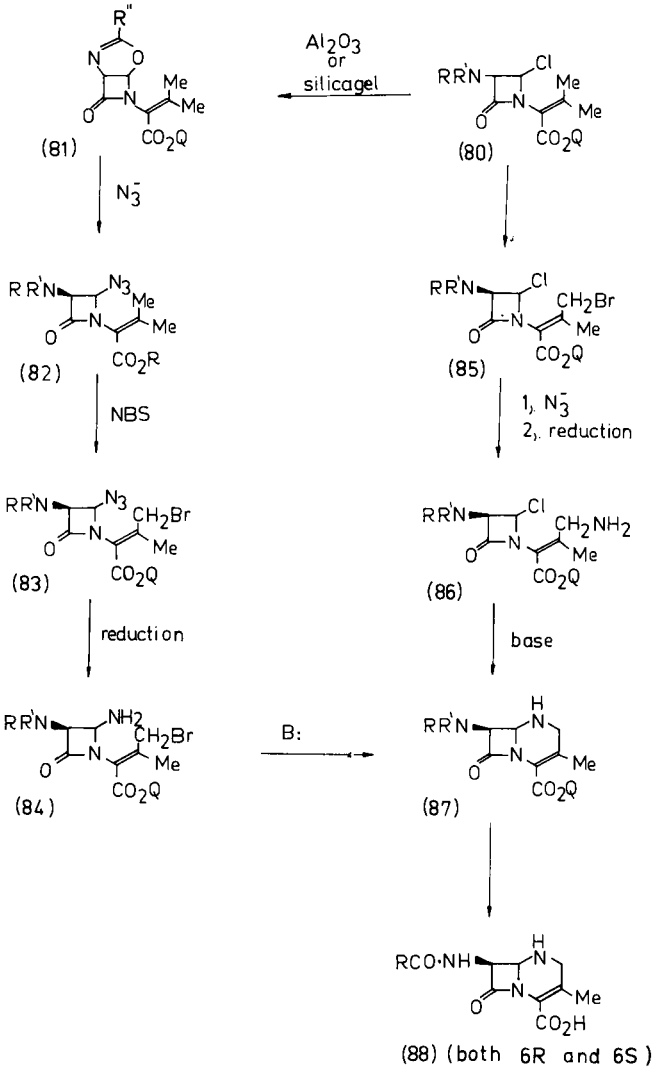
Starting with the chlorination of anhydropenicillins, both 6*S* and 6*R* derivatives can be obtained by any of the reaction sequences outlined on p. 196.

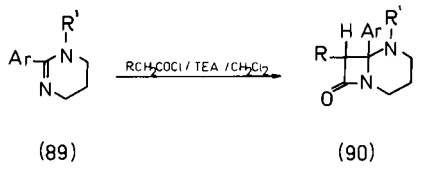
Compounds having usual side-chains, e.g. the phenylacetamido, phenoxyacetamido, α-amino-phenylacetamido and different isoxazole-4-carboxamido groups, have been reported [16], but no bioactivity data are available.

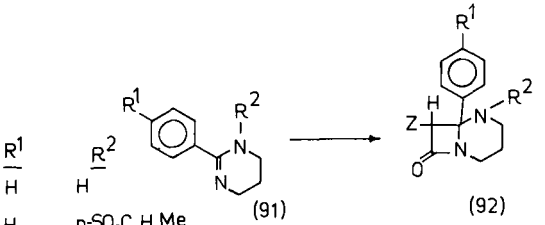
The 'acid chloride–imine method' [48] has resulted in numerous new β-lactams during the past decade [49,50]. In the attempted synthesis of 1-azaoctam analogues of cephalosporins, the *N*-acetylated tetrahydropyrimidines (89) were used as imine components [46] and different acid chlorides as ketene precursors in the presence of triethylamine (TEA).

This method is an extension of the known ketene–imine procedure, which has been in use for β-lactam formation since the beginning of the century [51]. Examples of this acid chloride → ketene route up to the end of 1971 have been reviewed by Mukerjee and Srivastava [52]. It is interesting to note that, depending upon the experimental conditions, both the concerted 1,2-cycloaddition path and another involving an acylation step and salt formation can be operative [53,54]. The former is supported by the formation of by-products of dioxopiperidine type [55,56]. Use of this procedure has provided a number of azaoctams of type (92) [46].

A similar cycloaddition is the stereospecific β-lactam synthesis from an acid chloride–base–thioimidate system [57]. This method has been employed successfully for the synthesis of closer analogues of cephalosporins than the previously-mentioned 1-azaoctams [58].


Al2O3
or
silicagel
(81)
(80)
N3-
NBS
(82)
(85)
1, N3-
2, reduction
(83)
(86)
reduction
base
(84)
B:
(87)
(88) (both 6R and 6S)

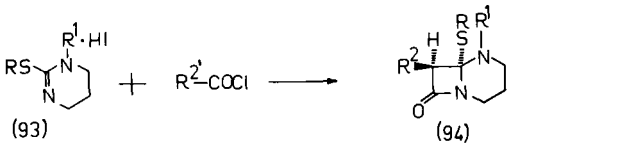

RCH2COCl / TEA / CH2Cl2
(89)
(90)



R^1	R^2
H	H
H	p-$SO_2C_6H_4Me$
CO_2Et	$COCH_2Ph$

(91) (92)

Z	R^1	R^2
OPh	H	$COCH_2OPh$
N_3	H	$COCH_2N_3$
NH_2	H	$COCH_2NH_2$
$NHCOCH_2OPh$	H	p-$SO_2C_6H_4Me$
OPh	H	$COCH_2NHCOCH_2OPh$
OPh	CO_2Et	$COCH_2Ph$



(93) (94)

R=Me, $PhCH_2^-$

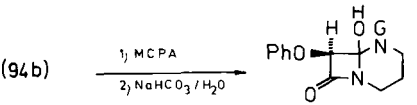
$R^{2'} \equiv R^2CH_2^-$

R=Me, $PhCH_2^-$

R^1= different acyl

R^2= PhO-, $PhCH_2O$-, N_3, NH_2, MeO, V, etc.

Compounds such as (94a) (1-phthalimidoacetyl-1-aza-6α-methylmercapto-7β-phenoxyacetamido-octam) are still without the 3–4 double bond and the free carboxylic group in position 4. These substituents are considered essential for acceptable bioactivity or even the lowest antibacterial activity level in most cases. It is noteworthy that the *S*-methyl group can be transformed to a hydroxy group by an oxidative process, by treatment with dilute aqueous sodium hydrogen carbonate solution [58]. Compound (95) is a unique β-hydroxy-β-lactam.



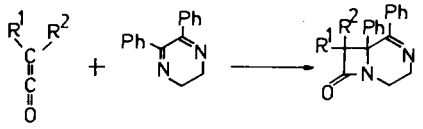
(94b) (95)

R=Me

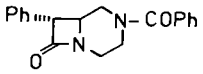
R'=G

$R^2 \equiv$ PhO

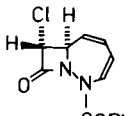
Ketene cycloaddition was employed for the preparation of a new 2-azaoctam derivative (98) [59]. Compound (99) is also a 2-azaoctam, but this compound is



(96) (97) (98)



(99)



(100)

a distant analogue of the known antibacterial bicyclic β-lactams [32]. A more strained system can be found in compound (100) [60]. This is 5-benzoyl-8α-chloro-5-azaonam-1,3-diene. The α-chloro substituent in position 8 provides good possibilities for obtaining derivatives bearing an 8β-acylamino side-chain.

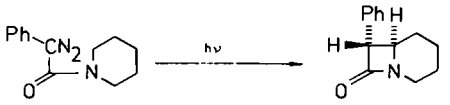
From the results of the attempted syntheses of aza-analogues of penicillins and cephalosporins, it is clear that the real breakthrough is still ahead in this particular field.

NUCLEAR ANALOGUES WITHOUT HETEROATOMS OTHER THAN THE β-LACTAM NITROGEN

Attempts to synthesize penicillin and cephalosporin analogues without heteroatoms other than the β-lactam nitrogen have been known from the literature for some time. These compounds are useful to illustrate that the sulphur plays no important role from the point of view of antibacterial activity. Similar compounds without antibacterial activity, however, underline the importance of certain structural requirements, which can be considered fundamental in the partial and total syntheses of these potential antibacterials.

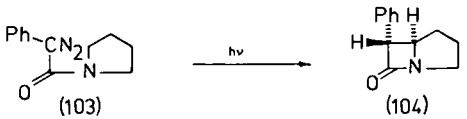
PENICILLIN ANALOGUES: HEPTAMS

Photolytic ring-closure of diazo-amides to β-lactams was observed by Corey and Felix [12]. Although they obtained the corresponding octam (102), their method is of general importance for the synthesis of new, fused β-lactam sys-

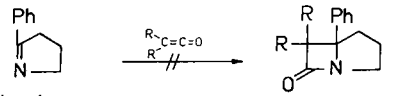


(101) (102)

tems. In fact, this synthetic procedure was employed for the production of the corresponding heptam (104) [43]. It is worthy of note that the ketene–imine cycloaddition procedure failed in the attempted synthesis of a similar heptam (106) [31]. Another synthetic route developed by the Lowe group was useful in

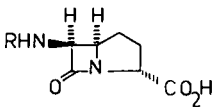


(103) (104)



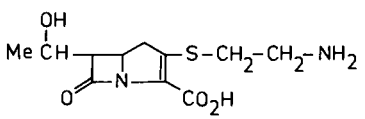
(105) (106)

the desired synthesis of the heptam (107) [11], which is a close analogue of penicillins. The key step in this synthesis is another photolytic reaction [65,66],



(107)

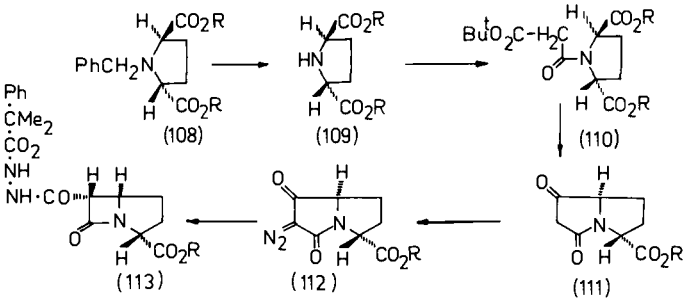
a modification of the Wolff rearrangement [61–64], which is successfully used for ring contractions in the case of alicyclic rings.



(107a)

Photolysis of the diazo compound (112) in the presence of phenylisopropyl carbazate at −70°C gave rise to the formation of the bicyclic trans-β-lactam (113) [11]. Because of the lability of the β-lactam of this compound even at room temperature, the synthesis was not completed as far as (107).

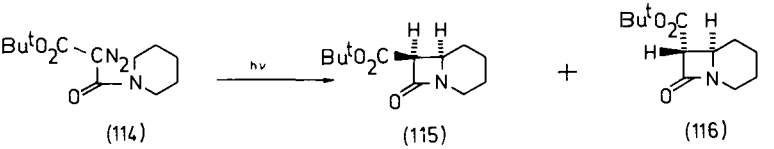
A new antibiotic, thienamycin (107a), has been isolated from the culture of



Str. cattleya NRRL 8057 by aerobic cultivation [66a]. It has broad-spectrum antibiotic activity against a range of Gram-positive and Gram-negative bacteria including *S. aureus, P. mirabilis, E. coli, K. pneumoniae, Ps. aeruginoase* and *E. cloacae* [66b].

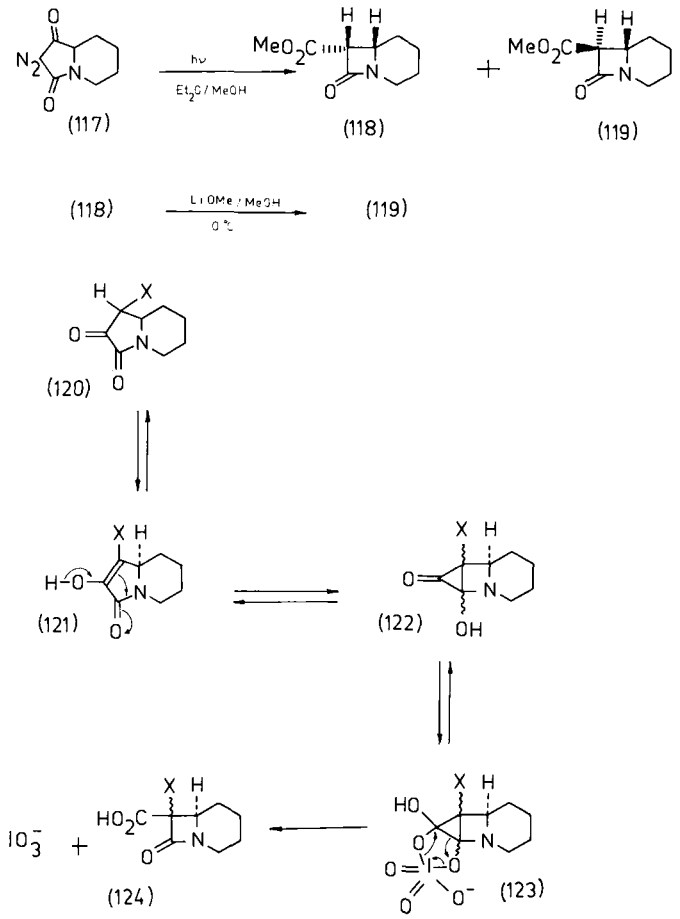
CEPHALOSPORIN ANALOGUES: OCTAMS

Following the method of Corey and Felix [12], Lowe studied the photolysis of the diazomalonic acid amide system. This modification is useful because it provides an ester substituent on the α-carbon of the β-lactam ring: a good target for further synthetic manipulations towards the desired amide substituent. From the first photolytic experiments of this system (114), both the *cis* (115) and



trans (116) stereoisomers were isolated [67]. This photolytic step is the key in the first synthetic route developed by the Lowe group.

The photolytic Wolff rearrangement gave the corresponding methyl esters (118) and (119) in a 2 : 5 ratio [68]. Compound (118), however, is rather sensitive to epimerization, even without addition of base, during storage at −10° [68]. Similar octams were found in an oxidative ring contraction of α-keto-γ-lactams (120) using periodate [69], and the mechanism proposed [70,71] is shown in (120)–(124).



An X-ray analysis of (124, X = Me) [71] gave its structure; the refined crystallographic structures were plotted stereographically using the computer programme ORTEP [72] (*Fig. 4.1*). It is well documented in *Table 4.3* that the β-lactam nitrogen bonding is not planar in bioactive systems. However, it is less pyramidal than the corresponding centre in tertiary bases [76] (e.g. in triethylamine the nitrogen is situated 0.56 Å above the plane determined by the three methyl carbons attached to it [88]). In amides, on the other hand, a planar structure can be found, and the nitrogen is in the plane determined by the substituents. From a compound having acceptable antibiotic activity, therefore, one might expect a pyramidal nitrogen which has a distance from the plane about midway between those for amides and tertiary bases (i.e. about 0.3 Å). In general, the corresponding β-lactam C=O stretching frequencies in the infra-red

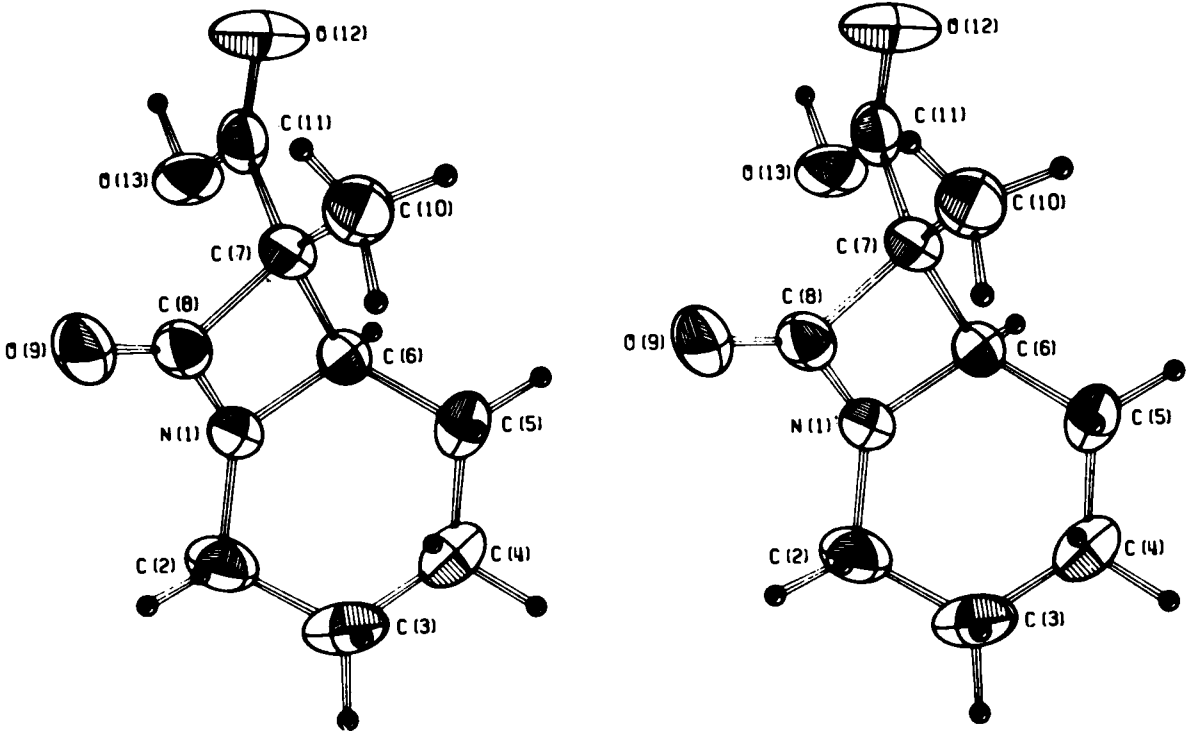


Figure 4.1. Stereoplot of the cephalosporin analogue (124, X = Me) [71]

Table 4.3. STRUCTURAL PARAMETERS IN CONNECTION WITH ANTIBIOTIC ACTIVITY FOR DIFFERENT β-LACTAMS [a]

Compound	*Distance of N from plane (Å)*	*Lactam C=O stretch (cm^{-1})*	*Antibiotic activity*	*References*
1. Penicillin G	0.40 [75]		+	
2. Penicillins		1790–1770	+	73, 74
3. Cephaloridine	0.24 [76]	Δ^3-cephems { 1776–1764	+	82
4. Cephaloglycine	0.22 [76]	Δ^3-cephems { 1792–1782	+	81
5. (±)Carbacephalothin [41]	≈0.3 [71]	1767	+	
6. Carbacepham G [79,80]	<0.09 [71]	1753 [b]	–	
7. Δ^2-Cephalorsporin V [74]	0.065	1760–1756	–	76, 82, 83
8. (125) [c] [77,78]	≈0.07 [71]	1784–1772	–	81
9. (124c)	0.089 [71]	1765	–	77
10. Unfused β-lactams [73]	0	1760–1730	–	73
11. Nocardicins [84–87]	0 (?)		+	87

[a] Data are partly taken from Refs. 76 and 83, where further details can be found in this respect.
[b] $\nu_{max}(CHCl_3)$ for the corresponding benzyl ester [80].
[c] (125), 7β-t-butoxycarbonyl-3α-benzyloxycarbonyl-4,4-dimethyl-2-thiaoctam.

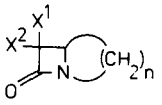
are higher for bioactive bicyclic β-lactam systems than those for inactive compounds [74,76,83]. There are compounds with higher β-lactam C=O frequencies and these data do not correlate with the bioactivity (e.g. penicillin sulphoxides, anhydropenicillin, epi-penicillins, etc.) [89,90]. These data are therefore of less value than those obtained from X-ray studies, at least as far as the pyramidal structure of the β-lactam nitrogen is regarded. In the light of some recent results, however, there are compounds possessing antibacterial activity, in which none of these requirements are fulfilled (cf. some monocyclic β-lactams [91–94] and the recently isolated antibacterials of the nocardicin group [87]).

The Corey method for preparation of different fused β-lactam systems [12,

Table 4.4. ACID HYDROLYSIS DATA OF SOME β-LACTAMS [96]

Compound	*HCl concentration ($M \times 10^4$)*	*Temperature (°C)*	*K × 10^4 (s^{-1})*
(126a)	3.85	30	11.06
(126c)	3.86	50	0.291
(126f)	3.65	50	0.126
	3.65	40	0.475

95] has been employed for the synthesis of different homocyclic fused β-lactams (heptams, octams, nonams) [96]. Compounds (126b) and (126c) have also been



(126)

	n	X^1	X^2
a)	3	H	Ph
b)	4	H	H
c)	4	H	Ph
d)	4	H	m-NO_2-Ph
e)	4	H	Me
f)	4	Me	Me
g)	4	Me	Et
h)	4	Et	Et
i)	5	H	Ph

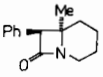
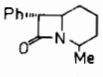
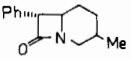
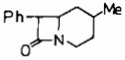
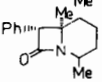
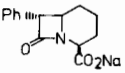
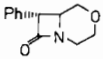
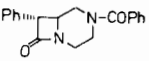
obtained by Moll (see later) and the latter by Corey and Felix [12]. Hydrolysis experiments [96] using acidic conditions have shown that the corresponding penicillin analogue (heptam (126a)) is much more sensitive to hydrolysis than the corresponding cephalosporin analogues (octams (126c,f)) and monocyclic β-lactam derivatives (*Table 4.4*).

These data are in good agreement with the observations of Lowe and Ridley [65,66], who encountered difficulties in the photolytic synthesis and purification of another heptam (113). Furthermore, the observed sluggishness of the corresponding octams in the attempted acid hydrolysis can be considered a result of the almost planar β-lactam nitrogen bonding in these systems [76]. On the other hand, these data show a tendency similar to that obtained by Moll

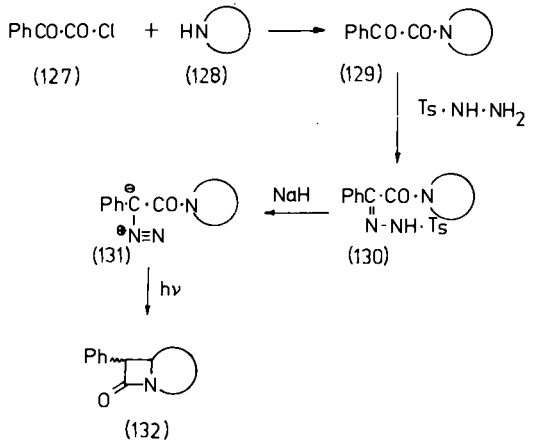
[97] and Feger [32] for similar systems. Moll obtained different simple octams, such as the unsubstituted octam (6) and the corresponding *cis*- and *trans*-7-phenyl derivatives (126b,c) [98–100]. This octam is also known from other experiments [101].

Feger [32] used Corey's method [12,95] to obtain a wide range of variously-substituted octams, oxa- and aza-octam, and a 6-phenylheptam (see *Table 4.5*).

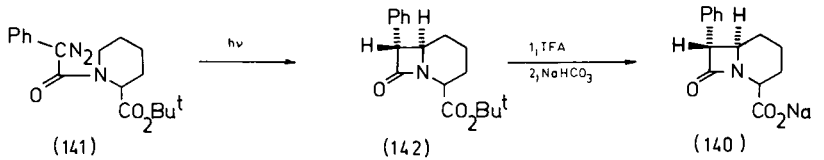
Table 4.5. BASE HYDROLYSIS DATA OF FUSED β-LACTAMS [32]

Compound	*Rate of hydrolysis* [a]
(133) 	$55 \cdot 10^{-5}$
(134) 	$26 \cdot 10^{-5}$
(135) 	$88 \cdot 10^{-5}$
(136) 	$55 \cdot 10^{-5}$
(137) 	$45 \cdot 10^{-5}$
(138) 	$16 \cdot 10^{-4}$
(139) 	$42 \cdot 10^{-5}$
(140) 	$17 \cdot 10^{-4}$
(50) 	$15 \cdot 10^{-3}$
(104) 	$26 \cdot 10^{-3}$
(99) 	$16.4 \cdot 10^{-2}$

[a] Hydrolysis data obtained in 0.5 KOH, 50°C by acidi–alkalimetric methods.

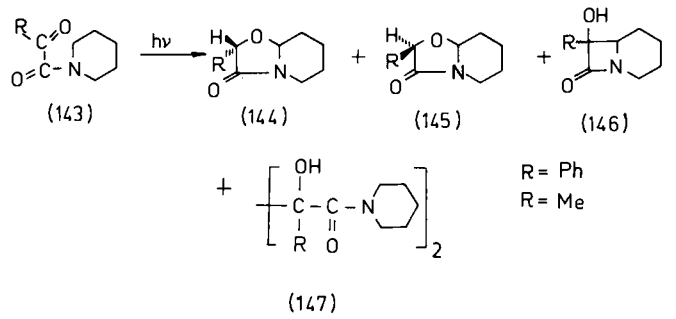


The base hydrolysis data for these compounds are listed in *Table 4.5.* It is noteworthy that the last four compounds exhibited markedly increased hydrolysis rates in comparison with Me- and Ph-substituted octams (133)–(139). Compound (140) was obtained using the Bu^t-ester-protected carboxylic acid [32].



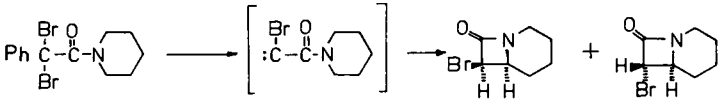
Because of the slightly pyramidal β-lactam nitrogen bonding, the *trans* stereochemistry of the β-lactam protons, and the 7α-phenyl substituent, no antibiotic activity can be expected in the case of (140).

Photolysis of β-keto-amide (143) gave the hydroxy-β-lactam (146) as a minor



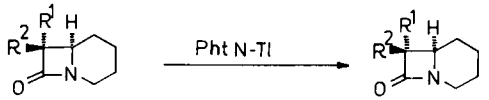
component [102,103]. In the attempted photolytic ring-closure reaction, the dimer (147) was also isolated [103]. A more fruitful method has been developed

for the synthesis of fused β-lactams by Johansson and Åkermark [104–106]. The method uses the thermolysis of a phenyl-mercury compound (148) to generate a carbene (149), which yields β-lactams (150) and (151). This method was successfully employed for the synthesis of penicillin analogues [107,108].



(148) (149) (150) (151)

The cyclizations yield mostly the more stable epi-derivatives (e.g. (151)), with *trans* stereochemistry of the β-lactam hydrogens. Nucleophilic displacement of the 7α-bromo substituent with thallium(I) phthalimide therefore results in the formation of the *cis* derivative (153) [109,110].



(151) R^1=Br, R^2=H,
(150) R^1=H, R^2=Br,
(152) R^1=Cl, R^2=H,

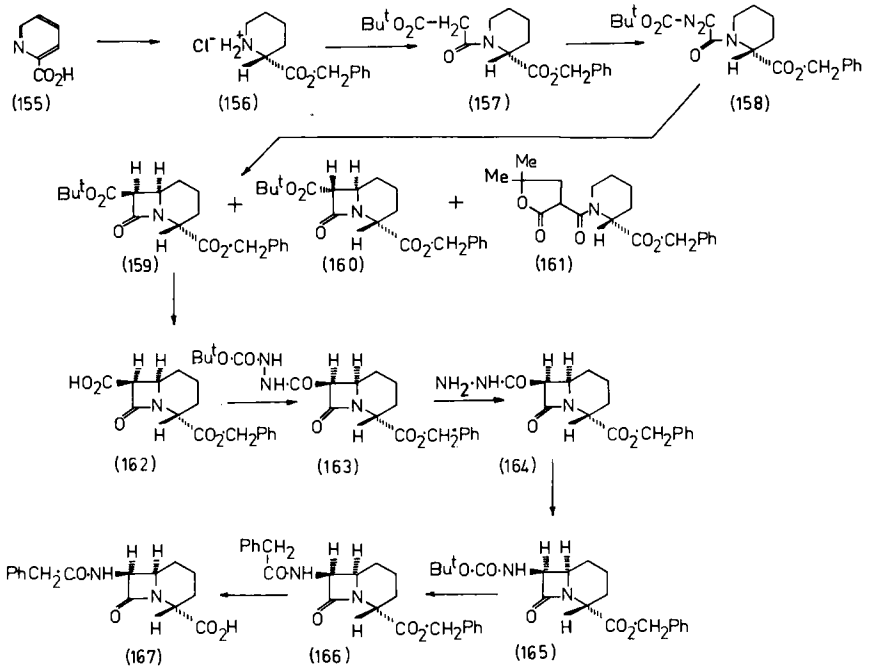
(153) R^1=H, R^2=PhtN,
(154) R^1=PhtN, R^2=H,

The general synthetic procedure (first synthetic route) developed for the synthesis of nuclear analogues of penicillins and cephalosporins by the Lowe group was used successfully in an elegant synthesis of 7β-phenylacetamido-octam-4α-carboxylic acid (167) [79,80]. The photolysis of the diazo compound (158) gave rise to the formation of the corresponding *cis*- and *trans*-β-lactams, (159) and (160), respectively. Treatment of (159) with base gave (160), indicating that there were no other differences in the stereochemistry of these octams. A minor component (161) was also isolated. In that case the carbene insertion took place on one of the methyl groups of the Bu^t-ester. Separation of the *cis* derivative by chromatogrphy gave the starting material of further synthetic manipulations leading to (167).

This nuclear analogue of cephalosporins was screened for antibacterial activity against three strains: *Alcaligines faecalis*, *Staphylococcus aureus* and *Salmonella typhy*, with cephalosporin C as control antibacterial agent. Activity was not observed at 1 mg/ml, nor a synergistic effect with cephalosporin C.

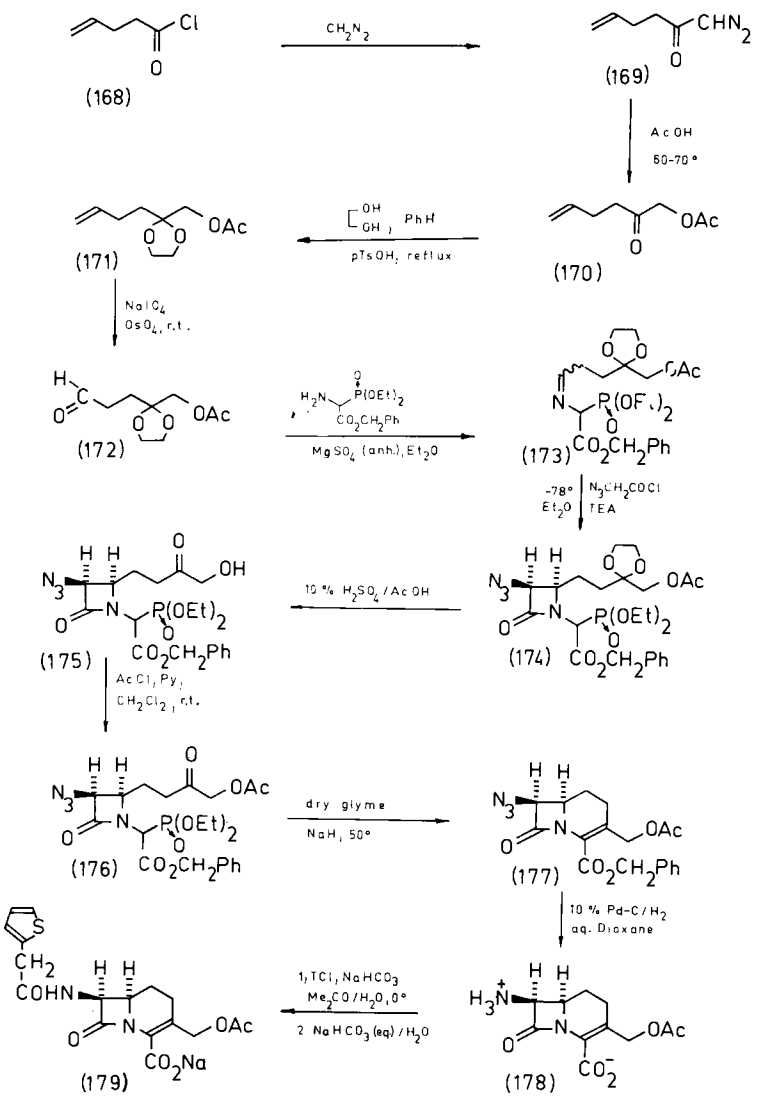
The only disadvantage is that the double bond found in bioactive cephalosporins cannot easily be introduced into (167), which would result in the formation of a more rigid system with a pyramidal nitrogen (see *Table 4.3* for details).

However, the method [80] developed for this synthesis can be used for the preparation of other analogues of the penicillin–cephalosporin group of antibiotics with more suitable steric and structural relations.



All the structural requirements are fulfilled in the Merck total synthesis of a cephalosporin analogue (179), (±)-1-carbacephalothin [41].

The synthesis started with commercial 4-pentenoic acid, which was converted to the acid chloride (168) with oxalyl chloride [111]. Diazomethane transformed (168) to the diazoketone (169), which was reacted with ethanol and then with ethylene glycol, giving (171). Oxidation of the double bond of (171) with sodium metaperiodate in the presence of osmium tetroxide gave the aldehyde (172) in 60% yield after column chromatographic separation. This aldehyde was condensed with the amine (52) to form a Schiff base, benzyl α-5-acetoxy-4,4-ethylenedioxypentanaldimino)-diethylphosphonoacetate (173). Cycloaddition of azidoacetyl chloride to this compound at −78°C resulted in the stereospecific formation of the *cis*-β-lactam derivative (174). Further manipulations gave (176), which was then cyclized with sodium hydride to the bicyclic product (177). Reduction and acetylation gave rise to the formation of the desired nuclear analogue of cephalosporins, (±)-1-carbacephalothin (sodium 3-acetoxymethyl-7β-thienylacetamido-oct-3-em-4-carboxylate) (179). Anti-



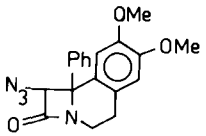
bacterial data for (179) are listed in *Table 4.6* [41]. These clearly indicate that the sulphur plays no important role in cephalosporins as regards the antibacterial activity of these compounds.

Table 4.6. MINIMUM INHIBITORY CONCENTRATIONS (μg/ml) OF (179) AND CEPHALOTHIN [41]

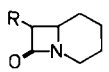
Strains	*Compound*	
	(179)	*Na–cephalothin* [a]
S. aureus 2865	1.56	<0.39
Str. pyrogenes 3124	<0.39	<0.39
Klebsiella sp. 2882	6.25	3.12
E. coli 2884	6.25	3.12
Shigella sp. 2880	6.25	3.12
Salmonella schottmuelleri 2837	3.12	3.12

[a] Racemic Na–cephalothin has approximately one-half the activity of 6(R),7(R)-Na–cephalothin [37].

A rather distant tricyclic analogue of cephalosporins, (180), was obtained by cycloaddition of a mixed anhydride to a bicyclic imine [112]. The synthesis of



(180)

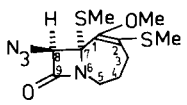


(181) R = GNH
(182) R = CO_2H
(183) R = CO_2Me

7-phenylacetamido-octam (181) has also been described [113] as well as other octams of type (126) [114,115].

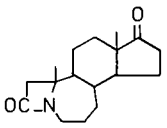
NONAMS AND NONEMS

A bicyclic nonem (184) was obtained in the mixed anhydride–imine cycloaddition procedure [116].



(184)

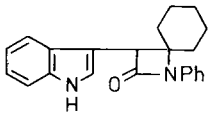
Another compound (185) and similar azasteroids have been synthesized [117–119], but these compounds are inactive as antibacterials [120].



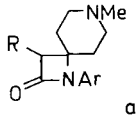
(185)

Spiro-nonams of type (186) [55] are of interest because of the anti-inflammatory activity observed in certain cases.

This anti-inflammatory activity was found when certain aza-analogues,

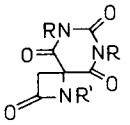


(186)



(187) a) R = N_3 b) R = GNH

(187a) and (187b), of (186) were synthesised [121,122]. The corresponding

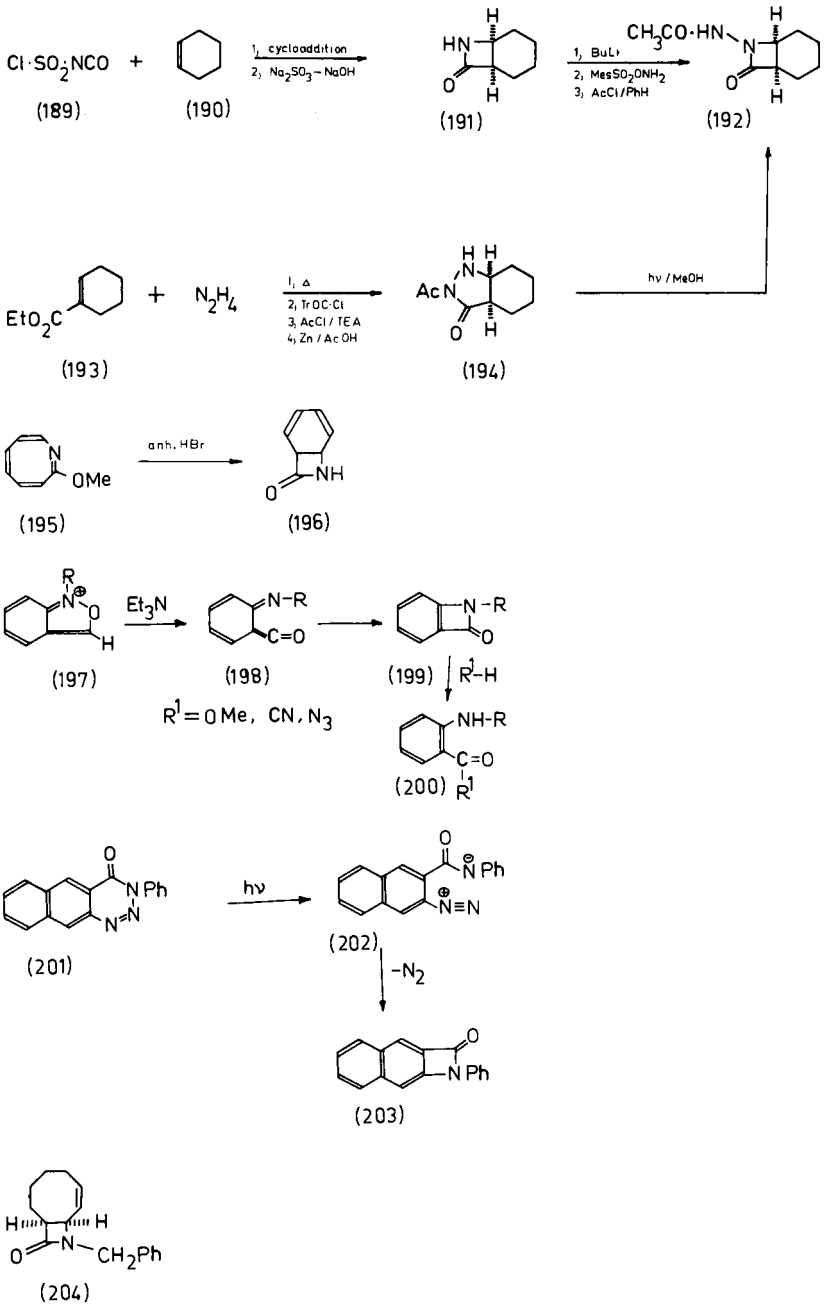


(188)

barbiturates (188), synthesised as potential CNS active agents, showed no activity [123,124].

NEOOCTAMS

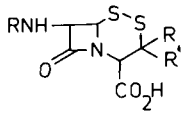
Neooctams, such as the unsubstituted parent compound (191), are easily obtained in cycloadditions. Isocyanates [125,126], sulphonyl isocyanates (43) [29,127] and especially chlorosulphonyl isocyanate (189) [128] react with alkenes to form variously-substituted β-lactams [129–135]. Synthesis of compound (191) is an example of this reaction which has been discussed in detail [52]. The same compound (192) can be obtained in the photolytic rearrangement of the pyrazolone (194) [136]. Compound (196) is neooctam-5,7-diene, obtained from the azocine (195) and hydrogen bromide [137]. Lactam (196) is a partly saturated form of (199). The latter is an unstable neooctam derivative, formed in an intramolecular ketene–imine cycloaddition reaction (see (198)) [138]. A similar compound ((199), R = Ph) was obtained by another method [139], while the corresponding tricyclic derivative, a naphtho-β-lactam (203), was formed in a photolytic process [140].


Cl·SO2NCO
(189)
(190)
1, cycloaddition
2, Na2SO3 – NaOH
(191)
1, BuLi
2, MesSO2ONH2
3, AcCl/PhH
CH3CO·HN
(192)
EtO2C
(193)
N2H4
1, Δ
2, Tr OC·Cl
3, AcCl / TEA
4, Zn / Ac OH
Ac N
(194)
hν / MeOH
OMe
(195)
anh. HBr
NH
(196)
Et3N
(197)
N–R
C=O
(198)
(199)
R1–H
R1 = OMe, CN, N3
NH-R
(200)
N Ph
(201)
hν
(202)
-N2
(203)
CH2Ph
(204)

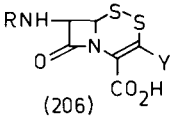
The previously-described neo-derivatives, as well as another neo-compound, 1-benzyl-5-neodecam (204) [141], are of less importance as potential antibacterials or synthetic intermediates than those containing heteroatoms in the ring attached to the β-lactam (e.g. (23), (38) and others described in the next subsection).

BICYCLIC ANALOGUES WITH MORE THAN TWO RING-HETEROATOMS

Nuclear analogues of cephalosporins containing two sulphur atoms (e.g. (205) [142,143] and (206) [143,144]) belong to this group, but some of their chemistry and properties were summarized in Part I. Highly-substituted 1-oxa-4-aza-

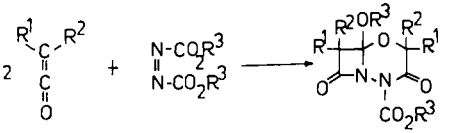


(205)



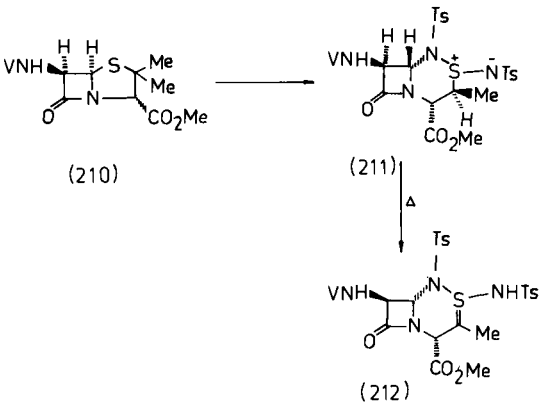
(206)

octam derivatives (209) were obtained in attempted ketene cycloaddition to (208) [145,146]. No bioactivity data are given.



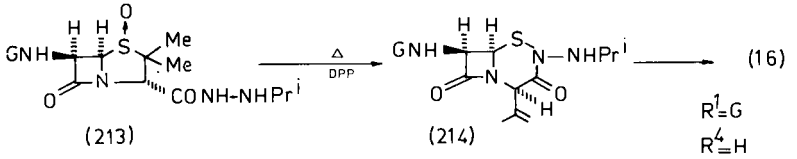
(207) (208) (209)

An interesting new reaction of penicillins with chloramine T gave rise to the formation of an ylide (211) [147] containing a substituted 1-aza-2-thiaoctam ring. Thermolysis resulted in the further transformation product (212) [148].



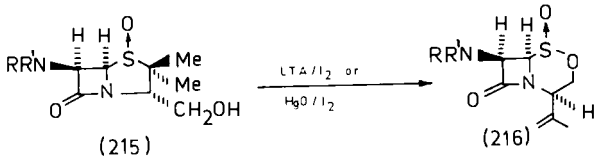
(210) (211) (212)

In an attempted ring-enlargement reaction, surprisingly, anhydropenicillin G (16) was obtained instead of 3-cephems [149]. In this reaction participation of a 1-thia-2-azaoctam derivative (214) was proposed, but this compound was not



(213) (214)

isolated. Similar 1-thia-2-oxaoctam derivatives (216) were obtained from the corresponding sulphoxides of the penicillanyl alcohol (215a,b) upon heating in refluxing benzene in the presence of either LTA/I_2 or HgO/I_2 [150].



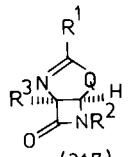
(215) (216)

a, RR'=Pht, α-sulphoxide
b, R=V, R'=H, β-sulphoxide

The α- and β-sulphoxides were obtained by this means, starting with the α- and β-sulphoxides of the corresponding penams (215a,b).

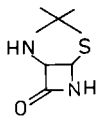
NEO-SERIES

Neoheptams and neooctams, containing at least two heteroatoms in addition to the β-lactam nitrogen, are far more important than the corresponding octams because these compounds are capable of undergoing several rearrangements, giving rise to the formation of compounds of antibacterial activity. Some representatives of these neo-derivatives can be found as synthesis intermediates in Part I of this review [161]. The previously-mentioned 5-oxa-7-azaoctams ((23), (38)) are of great synthetic importance in the partial and total syntheses of nuclear analogues of penicillins and cephalosporins.



(217)

Q=O, S, S-S, CMe_2, Me_2CS

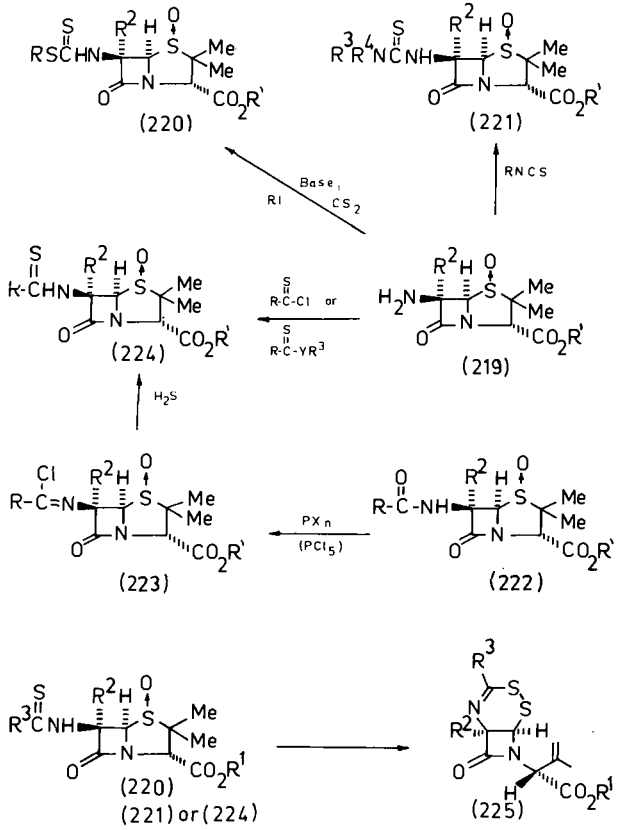


(218)

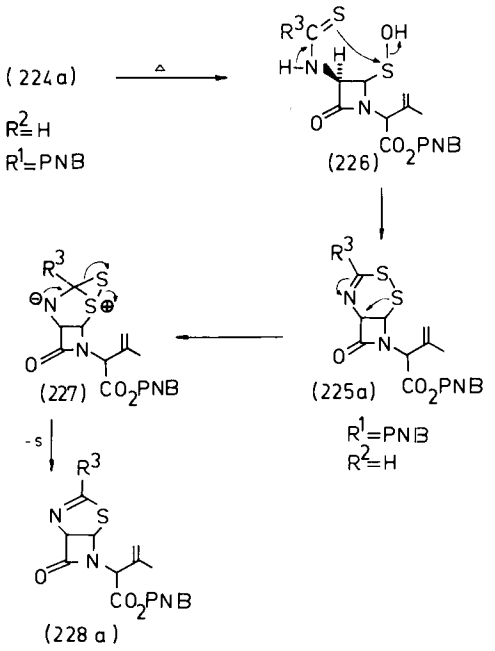
The exceptional synthetic utility of a saturated analogue (218) has been demonstrated in the brilliant total synthesis of cephalosporin C and analogues by Woodward [151–160]. The important work of CIBA-Geigy A.G. and the Woodward Forschungsinstitut in this respect has been discussed in Part I, and readers are referred to one particular reference [161]. For the chemistry of these useful synthetic intermediates (217) and (218), see some recent reviews [162–168].

An interesting transformation of penicillin sulphoxides to 5,6-dithia-8-aza-7-neoctem derivatives (225) is to be mentioned here.

The oxidation in good yield of 6-APA or its esters (219) [169], followed by thioamide formation ((200), (221) and (224)) [170–172] provides the starting



material for both (225) [172–174] and (228) [172,175]. In the formation of (225) a sulphenic acid intermediate (226) is possible, giving either (225a) [174] or (228a) [172] depending on the reaction conditions and substituents. The versatile transformation possibilities of the dithia-azaoctems formed (e.g. 225b)



have been reviewed, a variety of useful β-lactams being formed, depending on the amount of iodine used in the reactions [175].

Yields of the reaction (224) → (225) are almost quantitative, and further steps lead to 3-cephems in a high overall yield. 5-Thia-7-azaoct-6-em derivatives (228) also give 3-cephems upon treatment with HgO/I_2 [176].

β-LACTAMS OF NATURAL ORIGIN

Penicillins (1) were the first group of natural products found to contain a β-lactam ring. Other compounds from different sources in nature, however, have also turned out to contain a β-lactam ring, either in monocyclic form, or as part of a bicyclic system, as in the cephalosporins (2).

Cephalosporin C (236a) (see *Table 4.7*) was one of the constituents of a crude mixture of antibiotics obtained from a culture of *Cephalosporium* species by Brotzu [177]. The systematic work of Abraham in Oxford led to the isolation and structural studies of this compound [178]. Differences from the penam system were pointed out [179,180] and the proposed dihydrothiazine–azetidinone structure was confirmed by chemical [181] and X-ray methods [182]. This compound has also been obtained from several other organisms [183–185].

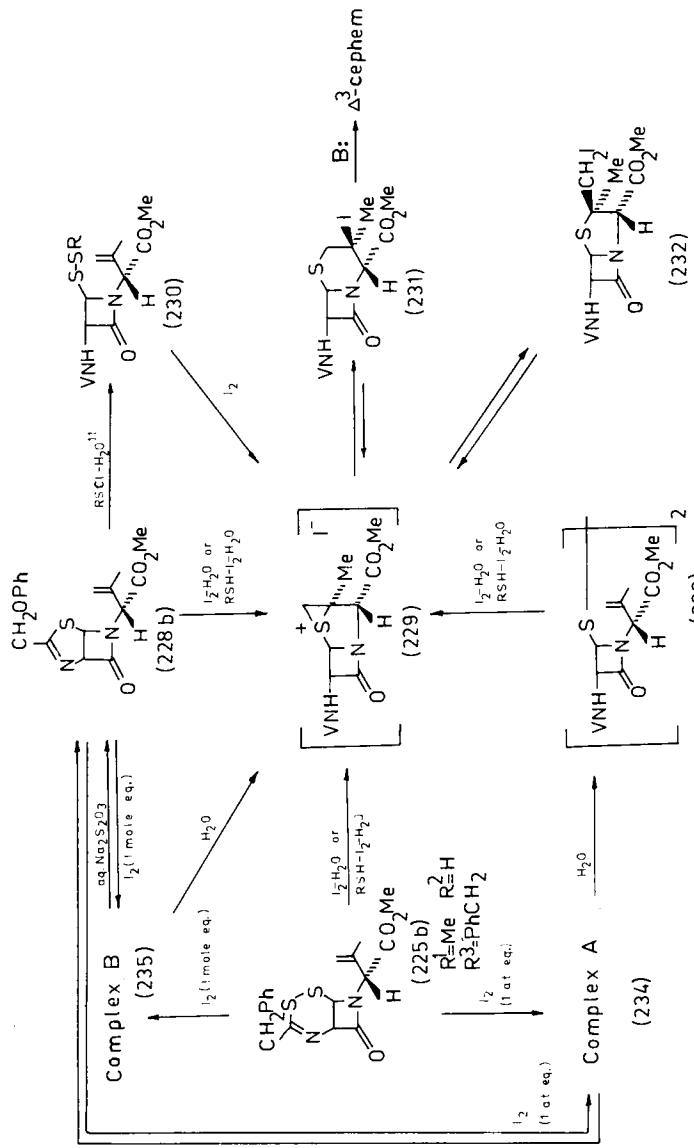
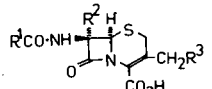
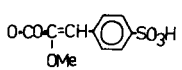
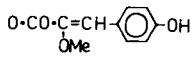
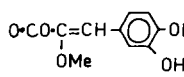

Complex B
(235)
aq. Na2S2O3
I2 (1 mole eq.)
I2 (1 mole eq.)
H2O
I2-H2O or
RSH-I2-H2O
(225b)
R1=Me R2=H
R3=PhCH2
I2
(1 at eq.)
aq. Na2S2O3
I2
(1 at eq.)
Complex A
(234)
H2O
(228b)
RSCl-H2O
(230)
I2
(231)
B: Δ3-cephem
(229)
(233)
(232)

Table 4.7. CEPHALOSPORINS OF NATURAL ORIGIN



(236)

	R^1	R^2	R^3	*Name*	*References*
a	$^{-}O_2C{\cdot}CH(NH_3^+){\cdot}(CH_2)_3-$	H	OAc	Cephalosporin C	177, 183–185
b	"	MeO	$O{\cdot}CO{\cdot}NH_2$	Cephamycin C	198
c	"	H	H	Deacetoxycephalosporin C	189
d	"	H	OH	Deacetylcephalosporin C	187, 188
e	"	H	lactone of	"	189
f	"	MeO	OAc	7α-methoxycephalosporin C	198
g	"	MeO		Cephamycin A	199, 200
h	"	MeO		Cephamycin B	199 200
i	"	MeO		C-2801X	201
k	"	H	SMe	TMC	198
l	"	H	$O{\cdot}CO{\cdot}NH_2$		202

The corresponding deacetyl and decaetoxycephalosporins (236d) and (236c) were isolated as metabolic products of different fungi and Streptomycetes. The deacetyl derivatives were also produced with citrus acetylesterase from cephalosporin C [186]. The compound shows reduced antibacterial activity compared to the parent compound (236a). This deacetyl derivative was produced by different *Cephalosporium* strains [187,188] together with other members of the cephalosporin family [189]. The deacetoxy derivative (236c), which is the reaction product of Pd–C reduction of cephalosporin C [190–192], is often a com-

Table 4.8. DEACETOXYCEPHALOSPORIN C-PRODUCING FUNGI AND ACTINOMYCETES [189]

Organism	*Culture number*
Cephalosporium chrysogenum	ATCC 14615
Cephalosporium sp.	NRRL 5445
Cephalosporium sp.	NRRL 5712
Cephalosporium sp.	NRRL 5716
Cephalosporium sp.	NRRL 5718
Cephalosporium sp.	NRRL 5719
Cephalosporium sp.	NRRL 5720
Cephalosporium sp.	NRRL 5721
Cephalosporium sp.	NRRL 5722
Cephalosporium sp.	NRRL 5723
Cephalosporium sp.	NRRL 5724
Cephalosporium sp.	NRRL 5725
Emericellopsis sp.	NRRL 5446
Emericellopsis sp.	NRRL 5447
Emericellopsis sp.	NRRL 5713
Emericellopsis sp.	NRRL 5714
Emericellopsis sp.	NRRL 5717
Paecilomyces carneus	ATCC 16329
Paecilomyces carneus	NRLL 2622
Paecilomyces carneus	NRLL 5711
Diheterospora chlamydosporia	NRRL 5728
Scopulariopsis sp.	NRRL 5715
Streptomyces lipmanii	NRRL 3584
Streptomyces clavuligerus	NRRL 3585

ponent of cephem-mixture metabolites of fungi [189] (*Table 4.8*).

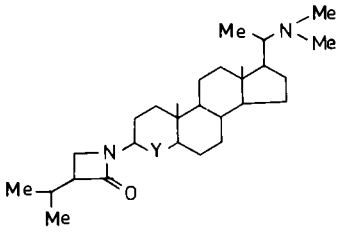
The occurrence of deacetoxycephalosporin C (236c) and the 7α-methoxy derivative of cephalosporin C (236f) provide an alternative route to the Morin method [14,74] for the preparation of cephalosporins of clinical usefulness. The side-chain exchange can be solved either using deacylation–reacylation methods [5,193–196] or via a diacyl derivative, successfully introduced for the preparation of cephamycin analogues [197].

The search at the Lilly Research Laboratories for new β-lactams of natural origin has led to the isolation of several new cephems bearing substituents other than acetoxy at C-10. During this programme, carbamoyloxymethyl-cephems (236l) and (236b) cephamycin C were isolated [198]. Other members of the cephamycin family (236g,h) [199,200] and (236i) [201] were found in different species of *Streptomyces* (see also ref. 183).

3-Methylthiomethyl-7β-(5′-amino-5′-carboxyvaleramido)-3-cephem-4-car-

boxylic acid (236k) is another natural cephalosporin [202]. This compound and similar 3-methylthiomethyl-3-cephem-4-carboxylic acids are available from synthetic sources too [203]. Murphy and Webber [204] have reviewed the development in the chemical substitution on the C-3 methyl group (C-10) of cephalosporins with a number of substituents, including various thio compounds.

Pachystermin A (237a) and pachystermin B (237b) are the only known representatives of natural steroid β-lactams [205].

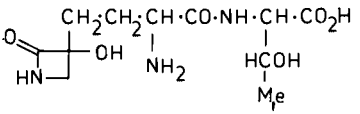


(237)

a. Y = >CO

b. Y = >CHOH

It is noteworthy that a peptide produced by *Pseudomonas tabacci* has also been isolated and found to contain a monocyclic *N*-substituted β-lactam ring (238b) [206]. This compound is the 'wildfire toxin' and the producing bacterium causes the 'wildfire disease' of the tobacco plant. Later it was re-named

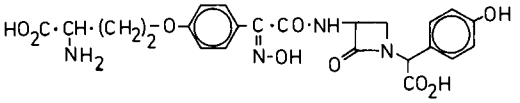


(238)

'tabtoxin' [207]. Another chlorosis-inducing *Pseudomonas* product (238a) is called (2-serine)tabtoxin [207]. A similar new compound has been described [116].

Compound (29) is another bicyclic β-lactam of natural origin, produced by *Streptomyces clavulerigus*. This compound, clavulanic acid, has already been mentioned among oxaheptams.

During the research programme of the Fujisawa Pharm. Co. in Japan interesting new monocyclic β-lactams (239) have been isolated from *Nocardia tsuyamanensis* ATCC 21806 [208]. This biosynthetic β-lactam antibiotic exists as *cis* and *trans* oximes (nocardicin A and B) and is unique from the point of view that it contains an unfused β-lactam system, a side-chain not previously found in biosynthetic β-lactam antibiotics. However, a new Glaxo cephalosporin, Cefuroxime, contains a side-chain similar to that in (239) [209].

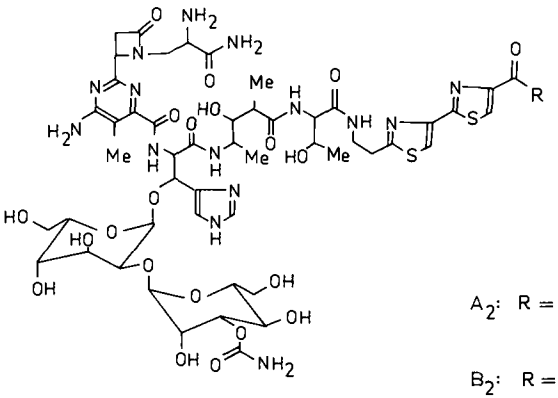


(239)

Another interesting feature of Nocardicin A is that it is active against Gram-negative bacterium strains, especially against *Pseudomonas*, but ineffective against Gram-positive bacteria. Nocardicin B is less active. β-Lactamases do not destroy them. These characteristics allow the hope that semisynthetic nocardicin analogues and monocyclic synthetic β-lactams of this type may be synthetised as potent, β-lactamase-stable antibacterials of practical usefulness to combat human infections caused by Gram-negative bacteria.

Streptomyces verticullus has been shown to produce a copper complex of an antibiotic phleomycin [210–212]. Later Bradner and Pindell [213] and Umezawa, Hori, Ishizuka and Takeuchi [214] reported the antitumour properties exhibited by this antibiotic. Falaschi and Kornberg have shown [215] selective inhibition of DNA polymerase by phleomycin. This compound, however, turned out to be toxic, causing irreversible kidney damage in dogs [216].

During the search for other, less toxic substances, another *Streptomyces verticullus* strain (B80-Z2) was isolated. This strain has been found to produce bleomycin A and B [217,218] (240). Detailed chemical studies [219–223] have shown bleomycins to be very close analogues of phleomycins [224]. Numerous



A_2: R = $HNCH_2CH_2CH_2S^{+}(Me)Me$

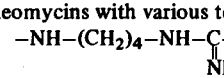
B_2: R = $HNCH_2CH_2CH_2CH_2HNC(=NH)NH_2$

A_5: R = $HN(CH_2)_3NH(CH_2)_4NH_2$

Bleomycinic acid: R = OH

(240)

Table 4.9. STRUCTURE–ACTIVITY RELATIONSHIPS OF BLEOMYCIN DERIVATIVES [236]

Compounds tested		*Strand scission of DNA* [a]	*Antimicrobial activity (μ/mg)* [b]
Bleomycins with various terminal amine parts			
$-NH-(CH_2)_4-NH-\underset{\underset{NH}{\|}}{C}-NH_2$	(Blm B_2)	100	3385
	(MOP -Blm)	113	592
$-NH-(CH_2)_3-N-Me_2$	(DMP–Blm)	100	810
$-NH-CH_2-\underset{\underset{NH_2}{\|}}{CH}-Me$	(DAP–Blm)	83	2178
$-NH_2$	(Blm–B_1′)	⩾100	685
$-OH$	(Bleomycinic acid)	5	159
$-O-(CH_2)_3-NH_2$	(Blm ester)	⩾100	1335
$-NH_2-(CH_2)_3-\overset{+}{S}-Me_2$	(Blm A_2)	⩾100	938
Other bleomycins			
Iso-Blm A_2		5	305
Phleomycin A_2 [c]		87	595
Pleomycin A_2 (Cu^{2+}-chelated)		<2	595
Epi-Blm B_2		⩾100	584
Enzymically inactivated Blm B_2		5	<300
Blm B_2 (Cu^{2+}-chelated)		<2	3480
Blm B_2 + Blm B_2 (Cu^{2+}-chelated)		⩾100	

[a] The *in vitro* activity (strand scission of DNA) was determined as described in [236]. The activity of each compound is expressed as that relative to the activity of bleomycin B_2 (100%).

[b] The antimicrobial activities were determined with *Mycobacterium* 607 as test organism.

[c] Phleomycin whose terminal amine is the same as that of Blm A_2 (tentative name).

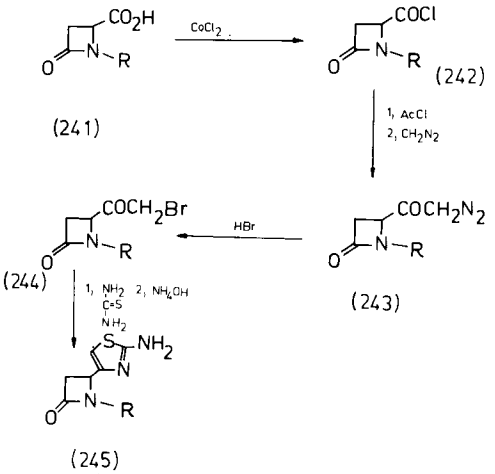
semisynthetic analogues of natural bleomycins are known [225,226]. Although the bleomycin structure is rather complex (240), it also contains a β-lactam ring. Bleomycins are antitumour and antibacterial substances [227]. In *in vitro* and *in vivo* experiments bleomycins cause fragmentation (strand scission) of DNA [228–235]. This effect on DNA is shown in *Table 4.9*, together with the antibacterial activity against *Mycobacterium* 607, used as test organism [236].

Structure–activity studies show a great variation, both in strand scission of DNA and antibacterial activity, with change in the chemical structure. The role

of the monocyclic β-lactam ring is rather obscure, and other parts of the molecule seem to have a great influence on the bioactivity of bleomycin (see *Table 4.9*).

MONOCYCLIC SYNTHETIC β-LACTAMS

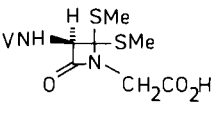
During the tremendous efforts to synthesize new active analogues of penicillins and cephalosporins, a vast number of various monocyclic β-lactams have been obtained, but alternative or improved methods of their syntheses are still being sought [52,162]. Particularly intriguing is whether these molecules possess antimicrobial activity, but in fact most of the available data reflect a complete lack of activity. For example, (245) easily made by the action of thiocarbamide on



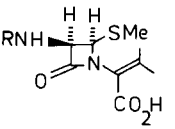
1-aryl-4-bromoacetylazetidinones [237], or (251), synthesized according to the second procedure [238] of the Lowe group, are typical members of this family. Similarly, no inhibition was found against usual test strains up to 1 mg/ml, for (252) [239] or 1,2-secopenicillins such as (253) [240–242].

Nevertheless, some activity against *B. subtilis* and *S. marcescens* has been attributed to (254)-like compounds, which anyway are valuable intermediates for total or partial synthesis of new penicillin or cephalosporin analogues (ref. 243 and the syntheses of the Sheehan group in ref. 35). More recently, the Bose group reported data on synthetic derivatives [91]. These compounds are available by treating the Schiff bases (256) with the appropriate acid chlorides in the presence of triethylamine. As shown in *Tables 4.10* and *4.11*,

(246)
(247)
(248 a)
(248 b)
(249)
(250)
(251)
hν



(252)



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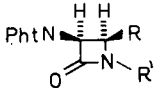
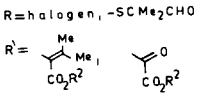
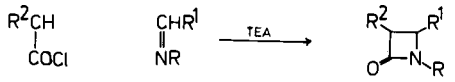




(254)



(255) (256) (257)

the MIC values of various (257) derivatives against various organisms lie between 25 and 100 μg/ml.

Table 4.10. SUBSTITUTED β-LACTAMS (257) [91]

	R	R^1	R^2
a	p-$MeCOC_6H_4$	p-$MeOC_6H_4$	OMe
b	Ph_2CH	p-$MeOC_6H_4$	N_3
c	Ph_2CH	p-$MeOC_6H_4$	NH_2
d	Ph_2CH	p-$MeOC_6H_4$	$NHCOCH_2Ph$
e	C_6H_{11}	o-$NO_2C_6H_4$	OMe
f	C_6H_{11}	o-$NO_2C_6H_4$	OPh
g	C_6H_{11}	o-$NH_2C_6H_4$	OPh
h	C_6H_{11}	o-$(NHCOCH_2Ph)C_6H_4$	OPh
i	p-$MeOC_6H_4$	2-furyl	N_3
k	p-$MeOC_6H_4$	2-furyl	NH_2
l	p-$MeOC_6H_4$	p-$(CO_2H)C_6H_4$	OPh
m	p-$(CO_2H)C_6H_4$	p-$MeOC_6H_4$	OPh
n	p-$(CO_2Me)C_6H_4$	2-furyl	OPh
o	p-$(CO_2H)C_6H_4$	2-furyl	OPh
p	Ph	o-$NO_2C_6H_4$	OPh
q	Ph	o-$NH_2C_6H_4$	OPh
r	Ph_2CH	p-$MeOC_6H_4$	OPh
s	Ph_2CH	p-$MeOC_6H_4$	OMe

The β-lactams (257o) and (257p) are of *trans* configuration, the rest have *cis* stereochemistry.

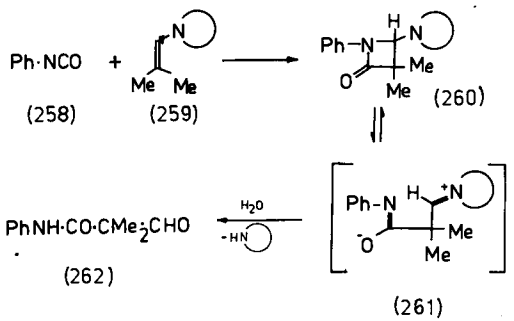
Table 4.11. ANTIBIOTIC ACTIVITY OF MONOCYCLIC β-LACTAMS (257) [91]

Compound	*Test organisms*	*MIC (μg/ml)*
(257a)	*B. subtilis* ATCC 6633	100
(257c)	*Str. haemolyticus* A 266	100
	Diplococcus pneumoniae L 54	100
(257e)	*Brucella melitensis* A 488 (Gram. neg.)	25
(257h)	*Brucella melitensis* A 488 (Gram. neg.)	25
(257l)	*S. aureus* ATCC 6538 P(A 55)	100
	S. aureus A 321	100
	S. aureus A 355	100
	S. aureus L 160 [a]	100
(257m)	*S. aureus* ATCC 6538 P(A 55)	50
(257o)	*B. subtilis* ATCC 6333	50
	S. aureus ATCC 6538 P(A 55)	50
(257q)	*Brucella melitensis* A 488 (Gram. neg.)	50
(257s)	*Brucella melitensis* A 488 (Gram. neg.)	50

A stock solution of the test compounds at a concentration of 2000 μg/ml in 0.05 molar phosphate buffer solution at a pH of 6.5 was prepared and two-fold dilutions were made with sterile buffer. 1-ml quantities of each dilution were incorporated into 19 ml of brain heart infusion agar in sterile petri dishes. The hardened surface was inoculated with the test organisms and it was incubated for 18 h at 37°C.

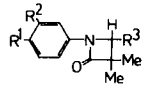
[a] The minimum inhibitory concentration (MIC), which is the least amount of compound completely inhibiting the test organism, was determined in μg/ml.

With the aid of a cycloaddition between isocyanates and enamines, the β-amino-β-lactams (260) were obtained by Abdulla and Fuhr [92]. Compounds (260) (*Table 4.12*) were found to be inhibitors of *Staphylococcus aureus* and



Bordatella bronchiseptica in the range of 10–100 μg/ml. It is worthy of note that quaternization of nitrogen in R^3 resulted in completely inactive compounds

Table 4.12. BIOACTIVITY OF SOME β-AMINO-β-LACTAMS (260) [92]



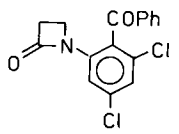
(260a-f)

Compound	R^1	R^2	R^3	*Strain*	*MIC (μg/ml)*
(260a)	H	CF_3	NMe_2	*S. aureus* 3055	10
				S. aureus 3074	10
				Str. fecalis X66	100
				Bordatella bronchiseptica	100
(260b)	H	Cl	NMe_2	S. aureus 3055	10
				S. aureus 3074	100
				S. fecalis X66	100
				B. bronchiseptica	100
(260c)	H	CF_3	1-morpholino	*S. aureus* 3055	10
				S. aureus 3074	100
				S. fecalis X66	100
(260d)	Me	Cl	1-morpholino	*S. aureus* 3055	10
(260e)	H	Cl	1-morpholino	*S. aureus* 3074	10
				S. fecalis X66	100
				B. bronchiseptica	100
(260f)	Cl	Cl	1-morpholino	*S. aureus* 3055	10
				S. aureus 3074	10
				B. bronchiseptica	100

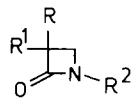
as were the formylacetaldehyde derivatives (262) obtained after hydrolysis of the β-lactams (260).

Mention must be made of certain 4-aryl-substituted β-lactams, which, when tested on a resistant strain of *S. aureus* in the presence of penicillin G, exhibited a marked inhibition of β-lactamase (*Table 4.13*) [244].

1-Adamantyl-2-azetidinone was found to have antiviral activity [245], but



(263)

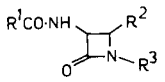


(264)

R=R^1=Pr^n R^2=Me

R=Ph, R^1=Et, R^2=H, Me

Table 4.13. BIOACTIVITY OF SOME 4-ARYL-SUBSTITUTED β-LACTAMS [244]



R^1	R^2	R^3	*MIC (μg/ml) of penicillin G against* Staphylococcus aureus MC_2					
	Inhibitor (μg/ml):		*50*	*25*	*12.5*	*6.25*	*5.12*	*0*
Ph · CH_2–	Ph	H	0.11	0.22	0.45	1.9	–	31.25
Ph · CH_2–	*p*-MeO·Ph	H	0.028	0.055	0.22	0.45	0.9	62.5
PhCH(N_3)–	Ph	H	0.055	0.055	0.055	0.11	0.9	62.5
p-$ClCH_2$·CO·NH·Ph·CH_2–	Ph	H	0.22	0.02	0.028	0.11	0.11	31.25
p-Cl_2CH·CO·NH·Ph·CH_2–	Ph	H	0.055	0.11	0.014	0.055	0.9	62.5
p-O_2N·Ph·CH_2–	Ph	H	0.028	0.028	0.014	0.028	0.055	62.5
2-Thienyl–CH_2–	Ph	H	0.055	0.055	0.11	0.22	0.45	62.5
p-H_2N·Ph·CH_2–	Ph	H	0.22	0.055	0.11	0.45	0.22	12.5
Ph·CH_2–	Ph	CHO	0.11	0.22	0.9	1.9	–	62.5

this is apparently due to the well-known adamantane moiety.

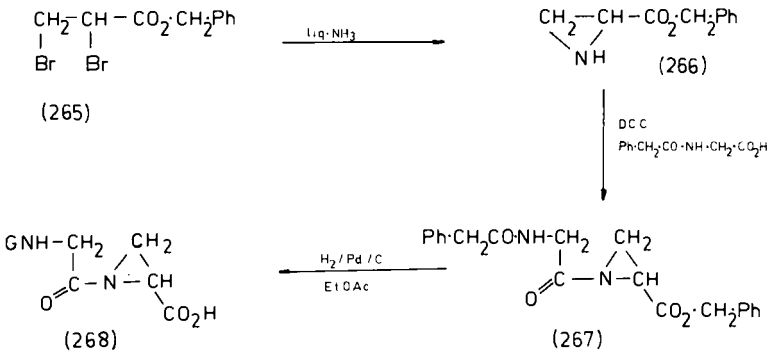
Anticonvulsant activity has been described in the case of β-lactam (263) (ED_{50} = 200–400 mg/kg) when tested against electroshock or metrazole-induced convulsion in mice [246].

The structural similarity of azetidinones with barbiturate-type tranquillizers led an Italian team to synthesize and screen β-lactams of type (264) for possible CNS activity [247–254]. These compounds showed pronounced sedative and tranquillizing activities in rats, mice and dogs, and may be categorized as minor tranquillizers.

DIFFERENT PEPTIDE-TYPE ANALOGUES OF PENICILLINS AND CEPHALOSPORINS

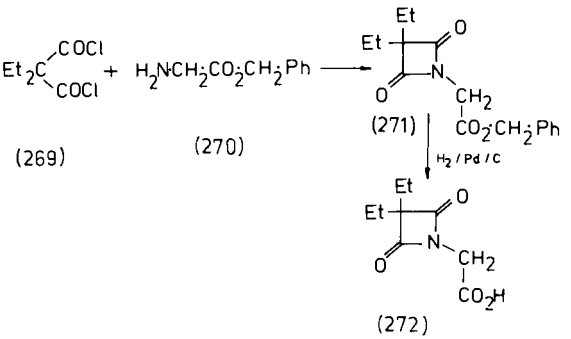
Starting from the assumptions that the antibiotic action of β-lactam antibiotics is due to their chemically highly-active β-lactam carbonyl group and, in turn, that the whole molecule can be regarded as a highly-strained dipeptide, a number of synthetic dipeptide-like compounds have been synthesized in the hope of their exhibiting antibacterial properties.

In one of the earliest attempts, Henery-Logan and Limburg reported the synthesis of *N*-phenylaceturylaziridine-2-carboxylic acid from benzyl α,β-dibromoproprionate [255]. Due to its aziridine ring, this compound may be considered



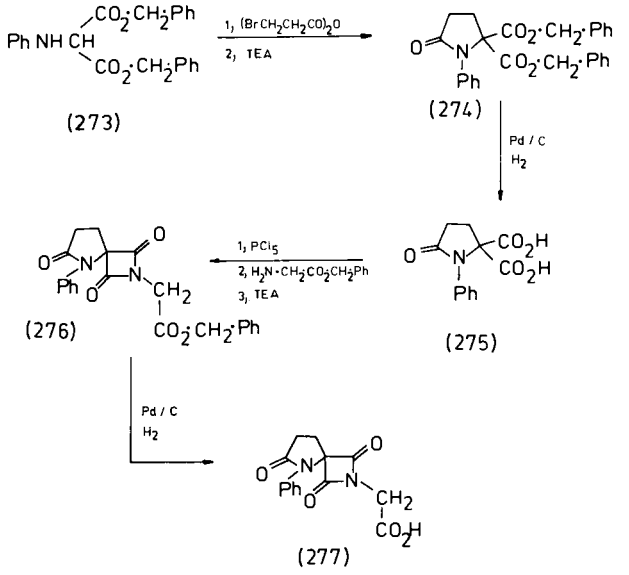
an activated acyl amine, but in the course of bioassay by serial dilution tests, its minimal inhibitory concentration was only 1–2 mg/ml.

The malonimido-acetic acid derivatives (272) and (277) also meet the requirement of a markedly activated peptide bond [256]. However, these compounds failed to reveal any worthwhile bioactivity. The same applies to compounds like



the γ-lactam homologue of penicillin [257] or succinimide derivative (283) (R^1 = Bz, R^2 = $-CH_2CH_2CONH_2$); the latter are available by two distinct routes. In screening experiments, (283) showed not more than one ten-thousandth of the bioactivity of penicillin G [258]. The necessity for the β-lactam ring is well demonstrated by (287), for the activities of these analogues lay in the range of 1/100–1/100 000 that of penicillin G [259].

The structural-analogy model of the mode of action of penicillins [260] has led to the syntheses of phenyl-acetylglycyl-D-Val-D-Val, -D-Ala-L-Ala, -D-Ser-L-Leu and certain similar peptides [261]. It was assumed that C-5 and S in the penicillins serve as the stretching points of phenylacetylglycyl-valine, as shown by (288); this stretching effect may be replaced by the template activity of transpeptidase, and thus a competitive antagonism could arise. Nevertheless, this con-



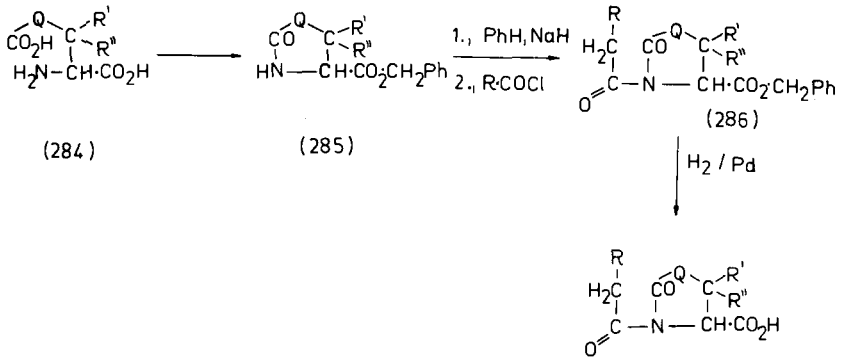
cept has failed, because the peptides in question do not show activity up to 2 mg/ml. These experiments reinforce the findings that a molecule with antibiotic properties has to meet the requirements of having both a reactive peptide bond and a suitable skeleton.

BIOSYNTHETIC ASPECTS AND BIOMIMETIC-LIKE SYNTHESES

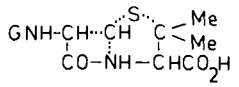
A great number of investigations have been made in attempts to shed light on the biosynthesis of β-lactam antibiotics. Nevertheless, as regards the most relevant question, little information has so far been obtained from the cyclization. The well-known feeding method is of little success, because only simple amino acids are able to enter into the micelial math. On the other hand, no cell-free system appropriate in every respect has been developed to date. The azetidinone ring system is rare in nature, although it is a characteristic moiety of pachystermins (237), wildfire toxin (238), bleomycins (240) and nocardicins (239).

On the results up to 1972 a very thorough review has appeared in the book by Flynn [2], which in addition deals with the biochemical background of the appropriate amino acids [262]. A newer review (1976) is that of Sammes [166] which sets out to consider the most probable and important points which can be gained from the ample results and which seem to confirm the tripeptide theory.

(278)
(279)
(280)
(281)
(282)
(283)

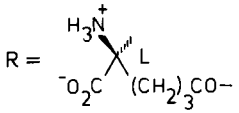


(284) (285) (286) (287)

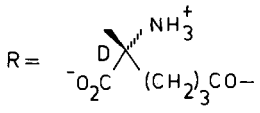


(288)

In the case of the *Penicillium*-type biosynthesis, the side-chain itself is highly dependent on the precursor used in the fermentation. In the absence of such a precursor, 6-APA can be separated [263–265], sometimes in the form of *N*-glycosides [266]. *Penicillium chrysogenum,* species of *Aspergillus,* some *Trichophytons* and *Epidermaphytons* belong to this group; the former micro-organisms produce isopenicillin N ((1), R = (289)) [267,268], the compound bearing an L-α-aminoadipyl side-chain.



(289)



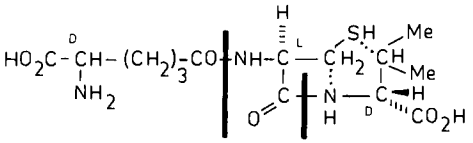
(290)

The *Cephalosporium*-type biosynthesis differs from the former in some points: the fermentation is independent of the given precursor acid [269] and no (or only traces of) 6-APA can be detected [270,271]. In contrast to the *Penicillium*-type biosynthesis, the corresponding 7-ACA does not appear as a product of fermentation. Members of this group are *Cephalosporium acremonium* (or by its newer name *Acremonium chrysogenum*) and *C. salmosynnematum* [272], synthesizing cephalosporin C ((2), R = (290), X^1 = OAc, X^2 = H) and penicillin N ((1), R = (290)).

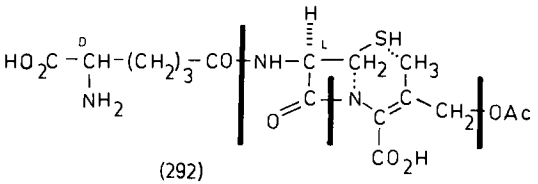
Various *Streptomyces* strains yield the important 7-methoxy-cephalosporin C

(236f), cephamycins (236b,g,h,i) and some other similar compounds (see *Table 4.7*).

The bicyclic skeletons of both the penicillins and cephalosporins can formally be divided into amino acid components, as shown by (291) and (292); this is of importance as regards the tripeptide theory.



(291)



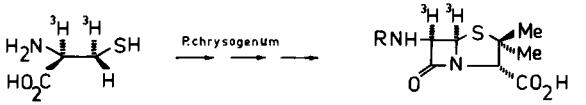
(292)

The tripeptide theory followed the isolation of L-α-aminoadipyl-L-cystein-L-valine (LLL-ACV or also called Arnstein-tripeptide) (291) by Arnstein, Morris and Toms [273,274]. Later LLD-ACV was found in strains of *Cephalosporium*-type [267,275,276]. Further support for the tripeptide theory emerged with the isolation of isopenicillin N, which could be regarded most probably as the common precursor of 6-APA and the various *N*-acylpenicillins [267,268,277, 278].

THE INCORPORATION OF AMINO ACIDS

L-α-Aminoadipic acid plays an essential role in biosynthesis, although in the assembled end-product it appears in the D form. Incorporation studies employing α-[6-^{14}C]aminoadipic acid show that the incorporation of the L isomer is considerably faster than that of the D isomer [279]. According to studies carried out on mutant strains of *Cephalosporium acremonium*, if the common lysine–L-α-aminoadipic acid metabolism is inhibited before the latter, then besides lysine the internal addition of α-aminoadipic acid is necessary to make the micro-organisms capable of synthesizing β-lactam antibiotics [271].

Investigations using labelled cysteine are of special interest in attempts to clarify the mechanism of ring-closure of Arnstein tripeptide. Because the label appears stereospecifically at the 6α and/or 5β positions (depending on the labelled compound used, see (293) → (294)), during the cyclization process

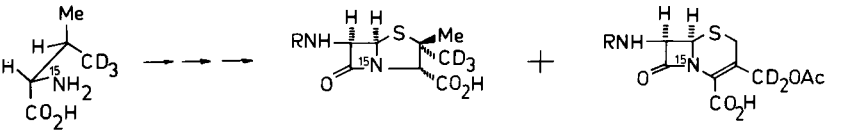


(293) (294)

intermediates such as α,β-dehydrocysteine (or 5,6-dehydropenam) are not probable, i.e. in the course of the cyclization cysteine shows retention of configuration [269,280,281].

As for the valine moiety, in the case of penicillins its C-1 (i.e. penicillin C-3) has D configuration ((291)). The existence of LLL-ACV and the isotope-labelled precursors suggest that the primary form is in the L configuration; the isomerization takes place after formation of the tripeptide.

The same applies to the *Cephalosporium*-type biosynthesis. L-Valine is also a cephalosporin precursor, although the valine moiety has a higher rate of oxidation because of its unsaturation and is also oxidized on C-10. Penicillin N and



(295) (296) (297)

cephalosporin C synthesized from (295) (or from any similar labelled compound) show that the isotope label appears in a strictly defined position. It follows from this that there is an inversion at C-1 of valine (i.e. LL L-ACV → 4-*D*-penam) but retention in position 2. The rates of isotope incorporation are similar in penicillin N and cephalosporin C which is further support of the existence of a common precursor. A further conclusion is that there is no possibility of a 1,2-dehydrovalinyl tripeptide intermediate (298), and thus stereo-

(298) (299)

specific cyclization, which proceeds very easily *in vitro*, is scarcely conceivable here [269,282–287]. However, the question of the 1,2-dehydrovalinyl intermediate (298) will be discussed later.

Oxidized derivatives of valine are not precursors; they presumably do not enter the mycelial math, and thus the functionalization of cephalosporins at

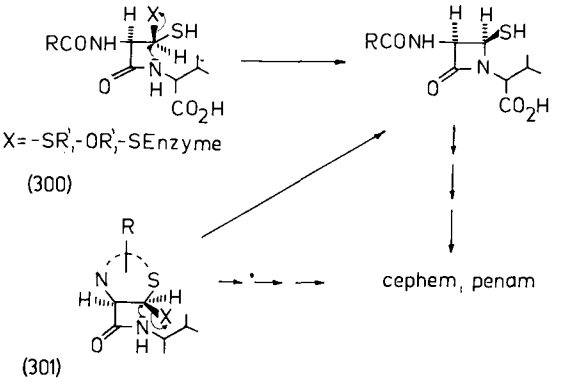
C-10 is a later step in the biosynthesis [288]. This assumption is supported by the fact that strains producing deacetyl and deacetoxycephalosporins have been found (see *Table 4.7*).

CYCLIZATION

It is not correct to separate the two cyclizations leading to the bicyclic system, because the possibility of a simultaneous process also exists; we do this for the sake of the *in vitro* experiments.

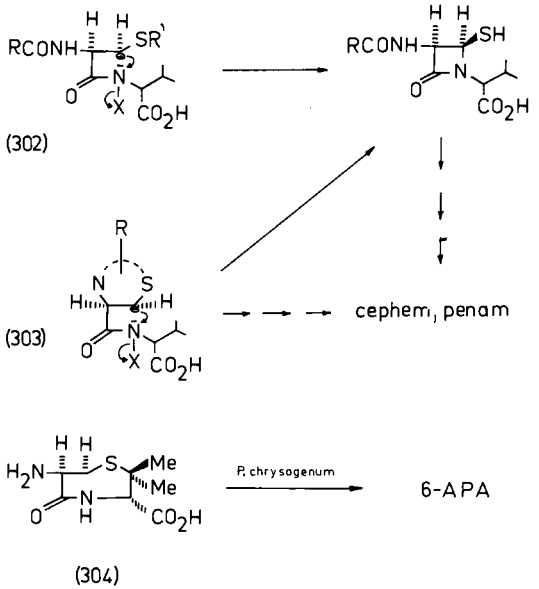
According to a recent paper [289], the penam 4–5 or cepham 5–6 bond may materialize in two ways (not regarding the theories presuming a 5–6 (6–7) double bond):

(i) Nucleophilic attack of amide nitrogen on the sp^2 carbon atom of a thiolaldehyde intermediate (300) (either on its sp^3 equivalent connected to some enzyme receptor or on a (301)-like thiazoline-azetidinone as suggested earlier by Cooper [290].



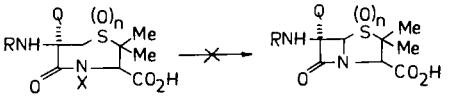
(ii) Oxidation on amide nitrogen followed by nucleophilic substitution of an anion generated on the β-carbon of the cysteine moiety ((302) or (303)).

It was thought earlier that 6-APA would arise from the cyclization of L-cysteine and D-valine [291,292]. There is no doubt that *P. chrysogenum* micelial math is capable of condensing cysteine, glycine and acetone to 6-APA in a reversible process [293]. The *in vivo* cyclization of (304) ^{14}C-labelled thiazepinone (cyclic L-cysteinyl-D-valine) has been investigated, but with negative results [294]. Newer *in vitro* experiments, shown in *Table 4.14*, also lack any success.



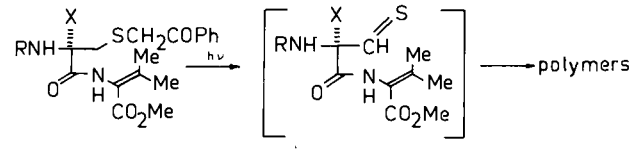
The thioaldehydes (308), mentioned under (i), can be obtained by photochemical methods. These are substances of high reactivity, but instead of the expected β-lactam product, only polymers were isolable [299]. On the other hand, it was assumed [300] that (309)-like compounds could rearrange to β-lactams via an 'ene' mechanism, but the photochemically generated (309) led to thiazolines (310) [301].

Table 4.14. EXPERIMENTS ON *IN VITRO* CYCLIZATION OF THIAZEPINONE DERIVATIVES

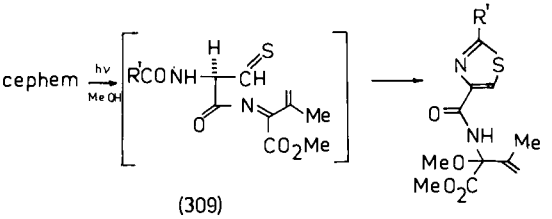


(305a-c) (306)

Compounds		*Reaction*	*References*
R = Ac, Q = Me, X = H, n = 0	(305a)	oxidation (Cl_2)	295, 296
R = Pht, Q = Me, X = Cl, n = 2	(305b)	Ag^+	297
R = $PhCH_2OCO$, Q = X = H, n = 0	(305c)	$h\nu$	298

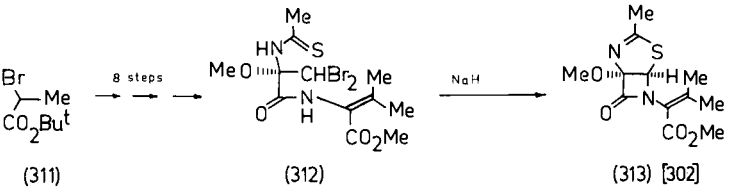


(307) (308)

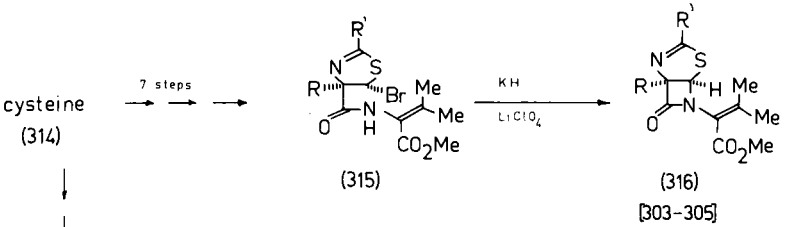


(309) (310)

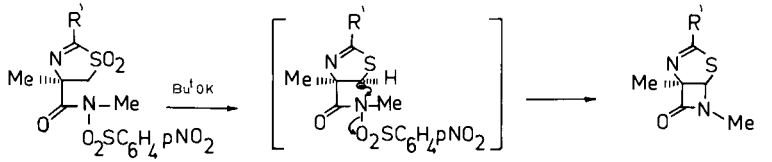
Cyclizations according to the assumptions of (1) and (2) are illustrated by sequences (311) → (313), (314) → (316) and (314) → (319). Although the thia-



(311) (312) (313) [302]



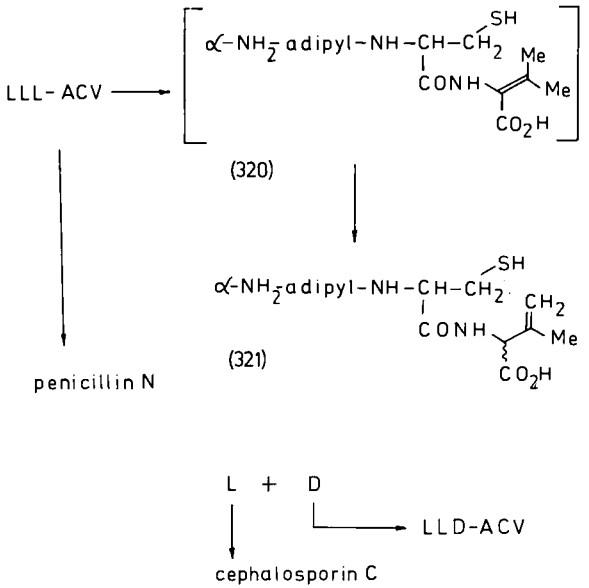
(315) (316) [303-305]



(317) (318) (319) [289]

zoline–azetidinone end-products are convertible to cephalosporins, the side-chain on the N atom should be unsaturated. This is not likely in the light of the previously-mentioned experiments with labelled valine.

Nevertheless, there is one possibility when a double bond-containing intermediate is conceivable (e.g. (298) or (320)) and the appearance of LLD-AVC in *Cephalosporium* strains could also be interpreted. It was already assumed [166] that in an early stage of the biosynthesis dehydrogenation of the valinyl moiety could take place to result in (321). If the reaction is not absolutely stereospecific



at the α-carbon atom of the valine, then after enzymatic reduction, a tripeptide with a D-valinyl unit would appear as by-product. The retention of configuration at the valine β-carbon atom according to (297) is acceptable by assuming that the consecutive reactions leading to (320) and (321) take place on the same active centre of a corresponding enzyme.

Present knowledge may be summarized as follows. After the appearance of LLL-ACV, the next compound isolated is isopenicillin N. This most probably isomerizes to penicillin N which, after enzymatic hydrolysis, serves as the source of different penicillins and probably 6-APA. In the case of cephalosporins, an 'isocephalosporin N' intermediate appears likely, although its existence has not been verified. Anyway, if *Cephalosporium*-type synthesis takes place, side-chain exchange does not occur, as the cells probably lack the appropriate enzymes. Recent problems regarding this pathway are that no definite enzymes have been found which are responsible for the isopenicillin N → penicillin N → side-chain

cleavage or transfer process [306,307], although an acyltransferase capable of transforming isopenicillin N has already been found in a *Penicillium* [308].

FUTURE TRENDS

In β-lactam chemistry, developments during the past few years can be considered revolutionary, as can be seen from the increasing number of patents, scientific reports and review articles which have appeared in this field. The successful partial and total syntheses of different true nuclear analogues of penicillins and cephalosporins, the isolation of monocyclic β-lactams possessing antibacterial properties, and the syntheses of monocyclic β-lactams with antibiotic activity, are all very important milestones of this development. Parallel to this, our understanding of questions involving structure–activity problems is becoming more thorough, providing the background required for the syntheses of new analogues of penicillins and cephalosporins with enhanced antibacterial properties. From the results gained so far in this field, in the near future one might expect powerful β-lactam antibiotics based on new ring systems from synthetic sources. However, semisynthetic possibilities utilizing compounds synthesized in soil microorganism laboratories provide important alternatives for obtaining improved antibiotics.

ACKNOWLEDGEMENTS

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5 The Role of Biogenic Agents in the Actions of Centrally-Acting Analgesics

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INTRODUCTION

The isolation of the alkaloid morphine from commercial opium and its subsequent introduction into therapeutics in the mid-nineteenth century as a pure, so-called specific analgesic agent, was a notable scientific milestone – not only because of the substantial improvement it represented in the treatment of severe and terminal pain, but also because the subsequent scientific study of morphine by medicinal chemists and pharmacologists has yielded many hundreds of active, useful derivatives of morphine, serving as models for the countless other pursuits and successes of the pharmaceutical industry during the last century.

Yet, the subcellular mechanisms by which the opiates and their derivatives exert their analgesic (and other) pharmacological effects remain largely unknown. The primary purpose of this review is to examine what is known of the interactions of opiates with central (and peripheral) neuronal transmitters such as noradrenaline (NA), dopamine (DA), 5-hydroxytryptamine (5-HT) acetylcholine (ACh) and histamine, in the hope that the subcellular actions of morphine may be better understood. Despite the many successes of the pharmaceutical industry referred to above, there remains a serious problem with analgesics in so far as analgesia, euphoriant effects and tolerance to (or dependence upon) the opiates largely remain inseparable properties; thus, new analgesics capable of combatting terminal pain always produce tolerance and physical dependence, whilst those devoid of addictive properties show themselves to be deficient in the control of chronic, terminal pain. It is to be hoped that, with a better understanding both of the pharmacological actions of morphine and of the loci at which these actions are profitably exerted, the search for safer, and still more specific, analgesics will be successful.

Chapters on the purely biochemical aspects, on structure–activity relationships and on recent developments in analgesic agonists and antagonists, have appeared previously in this series [1–3]. The purpose of the present review is to examine the extent to which the various properties of the centrally-acting analgesics can be explained in terms of alterations to the functional state of NA, DA, 5-HT, ACh and histamine.

CLASSIFICATION OF ANALGESICS

Although a relatively wide range of pharmacologically active agents, particularly certain psychotropic agents, may attenuate pain in animals and man under appropriate conditions, it is usual to classify as analgesics (or analgetics) only those agents which are used symptomatically and primarily to alleviate clinical pain. This is in contrast to the specific use of other drugs, such as antibiotics (for infections) or coronary dilators (for angina) which may alleviate pain through combatting the aetiology underlying the painful condition. Previous authors have classified analgesics in several ways, although a common device is to base the classification on their site or locus of action. In general terms, analgesics may be assigned to two basic groups which are composed of (a) peripherally-acting and (b) centrally-acting compounds.

PERIPHERALLY-ACTING ANALGESICS

Other authors have called these 'weak' analgesics, on account of the type and level of pain (often chronic in nature) for which they may most appropriately be

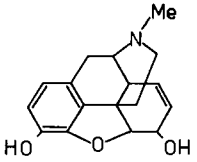
used. Peripherally-acting analgesics frequently possess anti-pyretic and/or anti-inflammatory activity as well, so that they find their chief clinical application in chronic rheumatic or somatic pain conditions. Their primary function is thought to be a control of the cause of pain, perhaps through modifications of peripheral inducing, transmitting or perceptive mechanisms of pain, rather than interference with man's awareness of, or reaction to, pain at a psychic (psychological) level [4]. Although the more commonly used agents may lead to an habituation of use, unlike the majority of centrally-acting analgesics they cause neither compulsive drug use nor physical dependence. Examples of anti-pyretic analgesics include the salicylates, notably aspirin; certain aniline derivatives such as phenacetin and paracetamol; and the pyrazole derivatives of which phenazone, amidopyrine and phenylbutazone are important examples. Other agents in this sub-group include indomethacin, mefanamic acid derivatives and the arylalkanoic acid derivatives such as ibuprofen.

Since the primary purpose of this review is to study the opiates and their immediate derivatives, it is not proposed to examine in detail the interactions of the weak, peripherally-acting analgesics with neuronal transmitters; readers interested in this group of drugs *per se* may consult reviews by Randall [4] and De Stevens [5]. However, this is not to totally dismiss as unimportant the role of central neuronal transmitters in the formulation and perception of some forms of chronic inflammatory or somatic pain; a number of recent reports have demonstrated the value of certain tricyclic anti-depressant agents in alleviating this type of pain in man [6,7].

CENTRALLY-ACTING ANALGESICS: OPIATES AND THEIR DERIVATIVES

The term 'opiate' was originally used to describe the alkaloidal constituents of the opium poppy, *Papaver somniferum.* The chemical structure of morphine (1), opium's major analgesic constituent, was not fully understood until 1923 [8] and confirmed unequivocally until 1952 [9]. Other authors have called these 'strong' analgesics to indicate their effectiveness against intense, perhaps visceral, pain of progressive pathology and life-threatening in nature, with usually a strong psychological component. Although it is possible to define two sub-groups, narcotic and non-narcotic, the vast majority of agents to be used successfully against clinical pain are close (or distinct) derivatives of the morphine molecule. Once the structure of morphine had been proposed, inevitably this was followed by considerable efforts to modify it and produce compounds of similar or greater potency, hopefully with greater specificity and without morphine's attendant disadvantages of respiratory depression and physical dependence. Whilst a satisfactory theory or model of central analgesic action still eludes physiologists and pharmacologists, significant gains have been achieved with a

number of morphine derivatives, the best known of which is pethidine (2), synthesized in 1939 by Eisleb and Schaumann [10].



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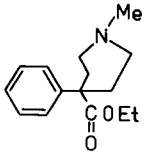
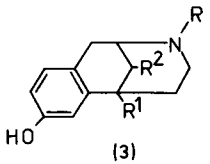


(2)

The structural relationship between morphine and pethidine was subsequently defined by Schaumann [11], when he suggested that the 4-phenyl-piperidine moiety was necessary for analgesic activity. With the advent of methadone and related agents, Schaumann [12] produced a more general theory in which he suggested that an aromatic ring attached to a quaternary carbon atom, two carbon atoms removed from a tertiary amine function, was an important basis of analgesic activity. As the list of new chemical structures with morphine-like analgesic activity grew longer, so the concept of the analgesic receptor was progressively modified [13,14]; in 1970, Casy concluded that present-day stereochemical and structure–activity data were incompatible with the concept of a single receptor defined by a rigid morphine skeleton. It is more likely that analgesics (and their competitive antagonists) bind ionically with a relatively simple, identical anionic centre which acts as a pivot around which a variety of modes of binding may occur [3,13]. At this point, it is pertinent to draw the reader's attention to the fact that both the catecholamines (as naturally-occurring amines) and amphetamine (as a synthetic amine) fulfil the simple requirement of the analgesic recptor in terms of structure and spacial configuration; under appropriate conditions, (+)-amphetamine is analgesic in several species including man, whilst noradrenaline may attentuate or enhance opiate analgesia depending upon the species studied and the amine's route of administration. On these grounds alone, it should be predicted that the opiates exert considerable effect upon aminergic function generally, a prediction supported by any consideration of morphine's wide-ranging pharmacological actions in the nervous system.

More recent developments have shown that the term 'opiate' (or narcotic analgesic) may be applied to a relatively wide range of compounds, and the classification is based more upon pharmacological properties than upon structural similarities; in fact, some of these agents possess structures not easily related to the general opiate concept, yet they exert qualitatively similar pharmacological effects to those of the morphine analogues. Other agents simultaneously possess a mixture of both narcotic agonist and antagonist properties (so-called partial agonists, [15]; for example, the Sterling–Winthrop team [16] synthesized and

studied a series of opiate antagonists based upon a 6,7-benzomorphan nucleus (3). One of these compounds, pentazocine (4), proved to be a useful analgesic agent since it was largely devoid of the psychotomimetic side-effects often elicited by other benzomorphans, yet it retained analgesic activity equivalent in potency to about half of that of morphine [17], with minimum addiction liability in man [18].



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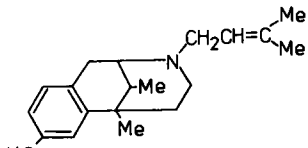


(4)

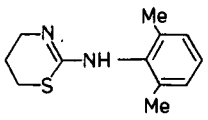
The term 'opiate', when applied to the partial agonist analgesics, is considered to be misleading because of the different properties and less stringent legislation applied to them; furthermore, some morphine-like analgesics are manifestly of different (non-opiate) structure. It is thus more appropriate to talk of 'narcotic analgesics' (agents with morphine-like properties, irrespective of structure), 'narcotic partial agonist analgesics' (drugs of the pentazocine type with mixed narcotic agonist and antoganist properties) and 'non-narcotic analgesics' (for other centrally-acting analgesics apparently interacting with the morphine receptor).

CENTRALLY-ACTING ANALGESICS: NON-NARCOTIC AGENTS

Fear, anxiety, depression and fatigue all tend to aggravate the human sensation and awareness of pain. Where these factors are reduced, the residual pain or suffering may be diminished by general analgesic therapy [19]. Thus it has been suggested that not only analgesics, but also certain psychotropic drugs, may be used in the treatment of pain. This suggestion has been substantiated at least in part by reports that tranquilizing drugs, such as those based on the phenothiazine nucleus, potentiate the action of narcotic analgesics [20] or have been used by themselves in the treatment of pain [21]. Similarly, antidepressant agents such as clomipramine [22], amitriptyline and the MAO inhibitor phenelzine [21], and even amphetamine [23] have been used to elevate pain thresholds. There is now a considerable body of clinical evidence that psychotropic drugs may be effective in the alleviation of pain. It is pertinent that all the drugs referred to above also interact in a fundamental way with the effects of one or more biogenic amines in the brain.

Other centrally-acting agents possessing potent antinociceptive activity include the sympathomimetic thiazine compound, xylazine (5) [24,25], and the

related sympathomimetic imidazoline, clonidine (6) [24]. These agents possess potent antinociceptive activity of a high order and may lack addictive liability, but they possess hypotensive and sedative side-effects at antinociceptive doses [26,27], which preclude their use as analgesics in man.



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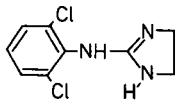
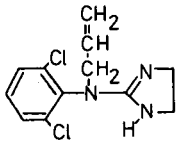


(6)

Further variations on the imidazoline nucleus may yield compounds with less potent hypotensive or sedative properties, whilst retaining other central sympathomimetic activity including the analgesic activity. An example of such a compound is St-567 (7) which displays a ratio of hypotensive activity to antinociceptive activity in rabbits some eight times more favourable than with clonidine [28].



(7)

It is to be hoped that still greater gains in analgesic potency relative to potency in other pharmacological tests will be produced. For the time being, the opiates and their close chemical relatives remain the major source of pharmacological relief from severe terminal pain.

Whilst animal studies are essential for the initial detection and full pharmacological evaluation of new analgesics, their eventual value as analgesics in man is not easy to predict. It was established long ago that experimental pain in man and clinical pain show different susceptibilities to drugs [29], so that the development of new analgesics will be inevitably beset with the problem of determining to what extent a new analgesic reduces that vital component or factor in clinical pain, the psychological reaction.

ASSESSMENT OF OPIATE ANTINOCICEPTIVE ACTIVITY IN ANIMALS

The evaluation of analgesic activity in laboratory animals has been reviewed comprehensively by several authors [29–32]. The pharmacological tests that are available rely on the application of an acute nociceptive stimulus to the animal,

with the subsequent measurement of the intensity or speed of the evoked response. Tests may be classified according to the types of analgesic drugs detected by each test or according to the nociceptive stimulus used. The ultimate value of the testing procedure must depend on the accuracy with which clinical analgesic activity is predicted. The most widely used animal tests employ chemical, electrical, mechanical and thermal stimuli.

CHEMICAL TESTS

In mice, intraperitoneal injection of certain irritant materials, notably phenylquinone [33], acetic acid [34], bradykinin, tryptamine, adenosine triphosphate, potassium chloride or acetylcholine [35], elicits characteristic abdominal 'writhing' or squirming syndromes, which are inhibited by pretreatment with a wide range of analgesic drugs. The test is simple to operate and requires no complex apparatus, but different strains of mice display marked variations in sensitivity to the stimulus [36], so that the experimental data must be interpreted with caution. Using phenylquinone, workers have shown that not only opiate agonists such as morphine, but also partial agonist analgesics, such as pentazocine, cyclazocine and nalorphine, exert clear-cut dose-related antinociceptive effects [37], their relative potencies correlating closely with those observed in man [38]. It has also been shown that the abdominal writhing test lacks specificity in so far as the writhing is inhibited by treatment with a wide range of pharmacological agents, many of which can be shown to be devoid of analgesic activity in man [39–41]. When used as a screening test, it produces a large number of 'false positives' which possess neither anti-inflammatory nor analgesic activity; yet the test remains useful for comparing the activities of already established analgesics of the narcotic partial agonist group [40].

ELECTRICAL TESTS

Electrical stimulation of the tooth pulp in dogs was first used as an antinociceptive test in 1938 [42], and the method has since been modified and used widely in the guinea-pig [43]. Nociceptive electrical stimuli have been applied to a variety of anatomical areas, including the rat scrotal sac [44], the mouse tail [45] and rectum of the rat [46]; in each case, vocalization by the test animal is used as the test end-point.

Other tests employing electrical stimuli have used behavioural responses as the end-point; an example is the rat tail stimulation test [47], when three separate thresholds may be identified with increasing stimulus. It has been proposed that this method is particularly useful for assessing the activity of the

narcotic partial agonists, and also that the third and final response of the animals (the vocalisation after discharge) requires a functionally intact thalamo–hypothalamo–rhinencephalic system. The test may therefore be analogous to the affective pain reaction in man [48].

The 'flinch–jump' test [49] employs an electrical stimulus applied to conscious rats through the floor (grid) of the cage in which they are housed. As the shock intensity is raised, the rats exhibit a 'flinch' or startle reaction, followed by a 'jump' response at higher intensities of stimulus. Both narcotic agonists and partial agonists increase the 'jump' threshold without affecting the 'flinch' threshold [50], and this represents an additional electrical test useful for predicting analgesic activity in opiates and related drugs.

Although electrical shock has been used widely as a nociceptive stimulus for evaluating opiate activity, problems do arise in controlling the intensity of stimulus; thus, electrode currents and voltages may be readily maintained whereas the impedance of the biological tissues can be extremely variable, thereby producing data of poor reproducibility [29,32]. The variability of data can be reduced when the animals can act as their own controls and also by chronically implanting electrodes subcutaneously sometime before the experiment so that impedance is minimised [51].

MECHANICAL TESTS

The 'tail clip' test was an early mechanical method used to assess analgesic activity. It involves the application of pressure to the tail of a rodent (using, for example, an artery clamp), the end-point being signified by vocalisation, biting or attempts to escape from the stimulus [52,53]. Whilst the test is usually evaluated on a quantal basis, (animal reacts (+) or fails to react (–) in stipulated period), the animal's responses may also be graded according to the speed of response. The test has the general disadvantage that the degree of pressure applied to the animal's tail is difficult to control precisely, and repeated testing on individual animals is impracticable because of the development of 'hypersensitivity' to the nociceptive stimulus.

A method employing the application of pressure to rats' hind paws was first described in 1957 [54]. The threshold pressure eliciting an escape response was determined when applied to a yeast-inflamed paw which was sensitive to touch [55]. The test is useful in detecting non-narcotic anti-inflammatory analgesics as well as the narcotic partial agonists (and agonists) [32,56]; the test can also be used with non-inflamed paws, when it is sensitive to narcotic agonist analgesics [57].

THERMAL TESTS

Thermal tests perhaps have been the most widely used in animal studies of antinociceptive activity. Available techniques may be classed according to the type of heat stimulus used, as for example radiant or conductive. The mouse 'hot-plate' test [58] and the rat 'tail-flick' test [59] are classic and widely used examples, in both cases a thermal stimulus being applied to a localised area of the animal and either the speed of response (qualitative assessment) or the presence or absence of a response (quantal assessment) determined. In the mouse hot-plate test, the end-point is characterised by paw withdrawal or paw licking after the animal has been placed on a hot (plate) surface, usually set at 55°C. A major disadvantage of the test is the inability to examine animals repeatedly because they rapidly condition to the stimulus; thus, animals may not be used as their own controls, and response–duration studies demand large numbers of animals. With the stimulus set at this temperature (55°C), only narcotic agonist analgesics reliably suppress the nociceptive response; other drugs (for example, barbiturates and neuroleptics) do so only in conjunction with substantial behavioural depression. When the plate temperature is lowered, for example, to 51°C, other centrally-acting drugs are then detected by the test, most important of which are the narcotic partial agonists [60–62]. However, in the present authors' hands, studies at the lower temperature yielded inconsistent and scattered data. In the rat tail-flick test, tail withdrawal times are measured in response to a radiated heat stimulus; an arbitrary 'cut-off' time, such as 15 s exposure, is imposed to reduce the chances of tissue damage, and with care this allows each animal to be tested repeatedly (say, at 30 min intervals) to determine the duration of antinociceptive effect. This method also is primarily useful for the detection of narcotic agonist analgesic activity, although, with care, activity can also be detected in the narcotic partial agonists and centrally-acting sympathomimetic agents such as amphetamine and clonidine.

The major purpose of this brief review of the more commonly used animal tests is to acquaint the reader with the techniques mainly employed in collecting the data reported later in this review; at the same time, some of the major disadvantages of the tests are indicated. With the exception of the phenylquinone writhing test which lacks specificity and is not suitable for repeated testing in individual animals), none of the small animal techniques is adequately sensitive to the non-narcotic centrally-acting analgesics.

Ben-Basset, Peretz and Sulman [63] described another thermal technique which involved a withdrawal response in mice after tail (or cord) immersion in a water bath maintained at 58°C [see also ref. 64]. Others have described a similar test using rats and a water bath at 55°C [65], whilst most recent work with a modified technique at 48°C (for mice) and 55°C (for rats) has demonstrated

dose-related sensitivity to narcotic agonists, partial agonists and other centrally-acting analgesics, with no interference from peripherally acting agents [66–68]. The data obtained by this technique are relatively precise, the test permits the repeated testing of animals so that response–duration studies can be carried out on individual animals, and interestingly the narcotic agonists and partial agonists produce characteristic but essentially different dose–response gradients. A range of psychotropic agents, for example, neuroleptics, minor tranquillizers and certain antidepressants, were inactive in this test at doses which did not substantially interfere with all types of behaviour [69]. This technique therefore has all of the advantages of the phenylquinone writhing test (sensitive to all centrally-acting analgesic agents) without the non-specificity of that test. Repeated testing of individual animals for up to 3 h after administration of drugs produces a stable control nociceptive sensitivity, and the test has proved important in the authors' studies of the interactions between biogenic agents and the actions of narcotic and non-narcotic analgesics.

POSSIBLE INVOLVEMENT OF BIOGENIC AGENTS

ACETYLCHOLINE AND NARCOTIC ANALGESICS

The involvement of a cholinergic component in the mechanism of action of morphine was proposed even before acetylcholine (ACh) had been shown positively to be present in the central nervous system. Slaughter and Munsell [70] demonstrated that the antinociceptive action of morphine in the cat was potentiated by concurrent administration of neostigmine, an inhibitor of the enzyme cholinesterase, a finding later confirmed in man [71,72]. Since then, a number of investigations have attempted to implicate ACh in morphine analgesia [73].

In 1957 it was reported that relatively low concentrations of morphine inhibited the release of ACh from electrically-stimulated guinea-pig ileum preparations, possibly through a post-ganglionic mechanism [74,75]; much later, a similar inhibitory effect on ACh release was demonstrated at the neuromuscular junction in the rat [76]. As a consequence, it was suggested that morphine acts at central cholinergic synapses by a similar mechanism, a concept extended during recent years to include a wide variety of narcotic agonists and partial agonists–antagonists [77–79]. Morphine also inhibits cholinesterase activity *in vitro* [80], although the extent of this inhibition depends on the enzyme source; more recently it has been suggested that cholinesterase inhibition does not constitute a major component of the pharmacological actions of morphine [81,82]. Nevertheless, in the presence of low doses of physostigmine, previously inactive

(non-analgesic) doses of narcotic partial agonist analgesics become active in the rodent tail-flick antinociceptive test [79]; further studies from the same laboratory, however, failed to show a correlation between the extent of brain cholinesterase inhibition and the antinociceptive activity of narcotic partial agonist analgesics [83].

Perhaps more significant have been the results of studies with cholinomimetic agents such as tremorine, its active metabolite oxotremorine, and physostigmine, each of which possesses marked antinociceptive activity in a number of laboratory techniques [79,84–91]. Confirmation that these are central effects was obtained by studies with oxotremorine given alone by intracerebroventricular (ICV) injection to mice, when it was found to be roughly equipotent with morphine administered by the same route [92]. Likewise, anti-muscarinic (atropine-like) drugs enhance the perception of pain and attenuate the antinociceptive effects of opiates in mice [93]. This evidence associates cholinergic mechanisms with antinociceptive activity in laboratory animals, although Sethy, Naik and Sheth [94,95] suggested that both the catecholamines (DA, NA) and 5-HT also play important roles in the mediation of antinociceptive activity by tremorine in mice. Thus, there may be some link between these three central amine transmitters and ACh with respect to antinociceptive activity.

A number of workers have reported that opiate analgesics elevate brain ACh levels [96–99], a property consistent with a central inhibition of cholinesterase, and tolerance to this particular action of morphine was observed when morphine was given chronically [98]. Others have been unable to show changes in rat brain ACh levels after acute [100] or chronic [101] administration of analgesic doses of morphine, although it was observed that morphine increased the 'bound' and decreased the 'free' ACh levels of brain homogenates [102]. Typically, the partial agonists have mixed (even paradoxical) effects upon brain ACh levels; when given alone, a range of narcotic partial agonists raised whole brain ACh levels yet reversed the morphine-induced elevation of brain ACh [79]. More recently, doubt has been cast on this dual action of the partial agonists [103].

The effect of opiates on the release of ACh from neuronal stores has been the subject of several detailed studies. Thus, morphine depressed ACh output from the cat cerebrum, whilst nalorphine and naloxone were inactive alone and antagonised the effects of morphine [104]. Similarly, the direct perfusion or intravenous administration of morphine suppressed the release of ACh from the perfused cat lateral ventricle [105]. Much of the *in vitro* work supports the *in vivo* studies; thus, morphine suppressed the release of ACh from cortical slices [99,106]. Furthermore, nalorphine, pentazocine and naloxone reduced ACh synthesis in rodent cortical slices (even though cyclazocine and oxotremorine increased synthesis under similar conditions [107]).

The possible involvement of a central cholinergic mechanism in the antinoci-

ceptive activity of analgesics has been studied using the ICV injection technique in conscious animals. A number of cholinomimetic agents have exhibited marked activity when given by this route [92,108–111], although physostigmine alone was not antinociceptive [112]. Dewey, Snyder, Harris and Nuite [113] designated a supraspinal area as the site of antinociceptive action by cholinomimetics in the mouse, a theory substantially confirmed by the actions of these agents when given by ICV injection; although inactive alone [112], physostigmine enhanced the antinociceptive effects of morphine, nalorphine and pentazocine when the former was given by ICV injection [114].

More recently, it was found that the antinociceptive activity of ICV-injected ACh was antagonised by atropine but not by the ganglion blocking agent, mecamylamine [115]. Also, ICV-injected ACh potentiated the actions of morphine, whilst partial narcotic antagonists reduced this action of ACh. The implications of these findings are that muscarinic-like receptors are involved at a central level in the antinociceptive effects of ACh and that this same mechanism may be participating in narcotic agonist (and partial agonist) analgesia.

ACETYLCHOLINE AND THE SUB-ACTUE AND/OR CHRONIC EFFECTS OF NARCOTIC ANALGESICS

From the evidence that morphine depresses both the synthesis and release of ACh in central cholinergic neurones, it was postulated that the development of opiate tolerance and/or dependence would be associated with a suppression of cholinergic activity [116]. Further, it was argued that the sudden removal of the morphine-induced restraint (for example, at morphine withdrawal) in dependent individuals would lead to a large 'rebound' release of ACh, producing withdrawal symptoms. Such a release of ACh has been demonstrated in rodents [117], and this has been accompanied by marked hyperactivity [118]. This line of argument is further supported by the observation that pretreatment of morphine-dependent animals with atropine reduces the severity of withdrawal symptoms, whilst pretreatment with the cholinomimetic agents, tremorine or physostigmine, exacerbated the withdrawal effects; further, the acute lethal dose of physostigmine was much lower in morphine-tolerant animals than in drug-naive animals [116], suggesting a sensitisation of receptors to ACh.

Although abrupt morphine withdrawal in dependent animals increases brain ACh levels for up to 6 h, withdrawal precipitated by the pure narcotic antagonist, naloxone, lowered brain ACh levels only in those mice and rats that were exhibiting the withdrawal jumping syndrome, and not in those animals that had failed to jump [117]; the conclusion drawn from these experiments was that the decrease in brain ACh (by the naloxone) was due to increased neuronal release during the withdrawal effects, and the effects themselves might be the cause of

(rather than be caused by) the release of ACh during the withdrawal period.

The many studies reviewed here support the general view that there is a cholinergic involvement in the brain in the antinociceptive effects of narcotic agonist (and partial agonist) analgesics. The majority of results suggest that the opiates impose some inhibitory effect on the release of ACh both *in vitro* and *in vivo*. Thus, a highly significant correlation has been observed between analgesic potency and the ability to inhibit ACh release [78], and also between clinical analgesic activity and the ability to depress contractions of the guinea-pig isolated ileum preparation [77]. How (or indeed whether) the antinociceptive activity *per se*, or the opiate-potentiating effects of centrally-administered cholinomimetics, correlates with the cholinergic inhibitory effects of the opiates still remains to be elucidated. In fact, there remains much work to be undertaken before any cohesive theory of the exact role of ACh (and cholinergic function in general) in the mediation of narcotic analgesic activity can be formulated.

THE CATECHOLAMINES AND NARCOTIC ANALGESICS

The presence of the catecholamines in the central nervous system (CNS) was first demonstrated in 1946 [119]. Several years later, dopamine (DA) was shown to be present in the brain [120,121] though unevenly distributed [122], whilst more recently, catecholamine-containing neurone systems in the CNS have been mapped out in considerable detail [123].

Vogt [124] demonstrated that morphine lowered brain levels of 'sympathin', and this discovery stimulated numerous further studies of the effects of the opiates on whole brain levels of catecholamines. Thus, it has been reported that single doses of morphine have no effect [99,125,126] or, in higher doses, either decrease [127–131] or increase [125,132] steady-state levels of brain NA. There has been a report that morphine increases brain NA turnover [133]. It has also been demonstrated that tolerance develops to the amine-depleting effect of morphine, and brain NA levels fall when morphine is withdrawn abruptly [127,128]. In contrast, other workers have detected no change in rat brain NA during chronic low-dose morphine treatment [125] whilst chronic high doses may raise brain NA [130]. It is not possible to draw any firm conclusion from these studies; a complicating factor is the wide range of species studied by different groups of workers under varying laboratory conditions. Changes in catecholamine levels or in catecholamine turnover occur following high single or chronic doses of morphine, but it remains to be determined whether these changes also occur at lower antinociceptive doses, and also whether the changes are the cause rather than the result of antinociceptive activity by the opiates.

Single doses of narcotic partial agonist agents, such as pentazocine, are reported to have no effect upon steady-state levels of both NA and DA, either in

rat whole brain or in selected regions [134]. However, another report [135] claims that pentazocine decreases steady-state levels of both DA and NA. Thus in this area, further work is required.

Until recently, relatively little attention had been paid to the effects of opiates on the synthesis and function of DA. However, a fall in brain DA levels has been observed in abstinent dogs [129], and also the time-course of morphine-induced antinociceptive activity correlates with a partial depletion of brain DA [136]. Furthermore, the partial agonist–antagonist nalorphine, at a dose which alone had no effect upon brain DA levels, has been shown to prevent these morphine-induced effects upon brain DA. It has been suggested that, where a fall in brain NA has been observed following administration of morphine, this was an indirect effect mediated through a reduction of brain DA, the latter acting as an inhibitory transmitter on spinal motor neurons involved in pain reflexes. Morphine-induced antinociceptive activity in the rabbit, using a tooth pulp method, is also believed to be mediated through DA [137], although there are reports that in both rabbit and cat acute doses of morphine do not alter brain DA levels [138]. When brain catecholamine synthesis was reduced in rats, using the tyrosine hydroxylase (EC 1.14.16.2) inhibitor α-methyl-*p*-tyrosine methyl ester (H44/68), the administration of morphine accelerated the depletion of brain DA [139,140], and it has been proposed that morphine increases the turnover of DA in ascending DA neuronal systems. In the same study [140], it was found that the abrupt withdrawal of morphine during chronic administration caused a decrease in DA turnover and an attendant increase in NA turnover in most brain areas, the latter phenomenon probably being caused by the stress of withdrawal.

There is now a substantial body of evidence that DA turnover is increased in the presence of narcotic agonist (and partial agonist) drugs. Thus, morphine increases striatal turnover of DA [140–145], as does methadone [145,146] and the partial agonist pentazocine [145]. Several groups have shown that morphine markedly increases the rate of DA synthesis at the tyrosine hydroxylase step [141,147–154], whilst inhibition of DA synthesis at this stage using α-methyl-*p*-tyrosine (α-MPT) potentiates some of the acute effects of morphine administration including its antinociceptive effects [152,155–164]. There is also evidence that, following morphine administration, there are increases in the brain levels of DA metabolites, presumably caused by an increased DA turnover in descending DA neurons [165]. There is thus an overwhelming number of reports that opiates in general increase DA turnover in the brain of several species. There are also data to locate this action of morphine on DA neurones centred in the corpus striatum; when these neurones are destroyed by the prior administration of 6-hydroxydopamine (6-OHDA), the antinociceptive action of morphine is substantially reduced [166]. This is an effect that can be reversed by the addi-

tional administration of the dopamine precursor, L-dihydroxyphenylalanine (L-DOPA), which it has been assumed is converted into DA by decarboxylase enzyme (EC 4.1.1.28), present in surviving dopamine neurones not destroyed by the 6-OHDA and/or by the same enzyme present in 5-HT neurones into which 6-OHDA is believed not to enter. More difficult to explain is the observation that the dopamine-receptor antagonist, haloperidol, potentiates the effects of morphine in mice [167] and enhances the development of tolerance. In animals already tolerant to morphine, there is an increased sensitivity to the locomotor stimulant action of L-DOPA. It has been postulated therefore that morphine exerts some of its central pharmacological actions by first interfering with DA-mediated synaptic transmission, and then initiating compensatory changes that have an overall resemblance to denervation supersensitivity; such a theory would certainly accommodate the observed changes in DA turnover made by previous workers.

Considerable additional data have been obtained by the use of other centrally-acting drugs. Thus, reserpine, an agent that causes a substantial depletion of both peripheral and central stores of both the catecholamines (DA and NA) and 5-HT [168,169], exerts a marked antagonist effect on the antinociceptive activity of morphine [170], a phenomenon that has been confirmed and extended by the present authors and other workers in several species, using a variety of nociceptive testing procedures [68,83,171–193]. Reserpine antagonises other pharmacological properties of morphine, including its ability to cause psychomotor stimulation [194,195] and produce lens opacity in rodents [196]. In contrast, there have been some reports that pretreatment with reserpine has no effect upon the antinociceptive activity of morphine [73,195,197,198], or may even enhance it [186,195,199–203]. There are several explanations for these conflicting findings, the most likely being differences in experimental conditions such as species of animals used, type of nociceptive testing procedure, and the duration of reserpine pretreatment. In the present authors' laboratories, reserpine treatment in both rats and mice transiently potentiated the antinociceptive effects of morphine [193], an enhancement which occurred at the time of maximum release of brain amines by the reserpine. It was proposed that reserpine can only potentiate the effects of morphine during this initial stage and that the major effect of reserpine pretreatment is an attenuation (or even abolition) of the antinociceptive activity of morphine, coincident with the prolonged amine depletion.

Tetrabenazine, a synthetic quinolizine with reserpine-like effects of shorter duration and of greater specificity for depleting central biogenic amines [204], has been used in a similar fashion to reserpine; likewise, pretreatment of laboratory animals with tetrabenazine substantially reduces the antinociceptive activity of subsequent treatment with morphine [174,178,205]. However, work with

reserpine or tetrabenazine does no more than indicate that interference in the central function of one or more of the amines DA, NA and 5-HT effectively abolished the morphine action. More selective agents are now available and these allow investigators to manipulate the functions of an individual agent (although data from this type of agent are not always unequivocal). α-Methyl-*m*-tyrosine (α-MMT) is reported to be a selective depletor of NA [206–208], and the use of this agent to pretreat rats [176] and mice [173] effectively antagonised the antinociceptive effects of morphine. Other workers have been unable to demonstrate this [187]. Sodium diethyldithiocarbamate (DDC) inhibits the enzyme dopamine-β-hydroxylase (EC 1.14.17.1) [209] responsible for the biotransformation of DA to NA, thereby facilitating the accumulation of DA in the tissues [210], whilst simultaneously reducing central NA concentrations [211]. Doses of DDC which significantly reduce brain NA levels were found to potentiate the antinociceptive activity of morphine, both in mice [212] and rats, although in the latter species there was not a good temporal correlation between enhancement and altered brain levels of DA and NA [213].

The selectivity of an agent can be markedly improved by injecting it directly into the brain, for example, by ICV injection or by stereotactic injection into specific areas of the brain. Thus the agent 6-OHDA given by ICV injection produces a selective degeneration of catecholamine-containing neurones in the CNS [214,215]. When morphine was given to animals pretreated with ICV 6-OHDA, there was a significant increase in the level of antinociceptive activity [216,217], an effect attributed to destruction of adrenergic neurones in the medial hypothalamic nuclei.

A still more direct and selective approach to the problem of determining catecholamine involvement in opiate antinociceptive activity is to give these amines by ICV injection, either alone or in animals also given an opiate by conventional (peripheral) injection. In this way, the blood–brain barrier to the amines is largely circumvented because of the relatively high concentrations of amine that can be achieved within the ventricles and adjacent periventricular grey tissue; the ICV technique also largely prevents direct cardiovascular changes which these agents produce and which might interfere with basic nociceptive sensitivity. Although an initial study showed ICV-administered NA to be antinociceptive in the mouse hot-plate test [92], later studies have shown that ICV NA does not significantly alter nociceptive sensitivity in several testing procedures in either the mouse or the rat, but markedly attenuates the anticnociceptive effects of morphine [114,193,218], other narcotic agonist analgesics such as diamorphine, etorphine, pethidine and AH 7921, and narcotic partial agonists such as cyclazocine, nalorphine and pentazocine [66,68,219]. This suggests that NA may have a positive modulatory role in the transmission of nociceptive impulses (and a negative one in opiate analgesia). On the other hand, ICV injection of low

doses of DA potentiated the antinociceptive effects of morphine in rodents [220], although higher doses of ICV DA also antagonised the effects of morphine; the latter effect was attributed to NA being formed *in vivo* from the larger doses of DA, so that it was concluded that DA and NA have opposing effects with respect to opiate analgesia (or the transmission of nociceptive impulses in the periventricular tissues).

Drugs possessing α- and β-adrenoceptor blocking activity (for example, phentolamine, phenoxybenzamine, propranolol and practolol) have also been studied in connection with opiate analgesia. When administered by a peripheral route, the α-adrenoceptor antagonist, tolazoline, antagonised the antinociceptive activity of morphine [175,176], whilst two other α-adrenoceptor antagonists were antinociceptive themselves and also enhanced the activity of morphine [221]. Initial work with the β-adrenoceptor antagonist, propranolol, showed an antagonistic action against the antinociceptive action [222] and other morphine-induced behaviour [223], but more recently other workers [221] concluded that neither propranolol nor practolol affected the antinociceptive activity of morphine.

The adrenoceptor antagonists have also been studied following their ICV injection. Thus, phentolamine (α-blocker) and propranolol (β-blocker) both exerted antinociceptive activity alone [224]; when given in combination with morphine, ICV phentolamine, but not propranolol, substantially enhanced the antinociceptive effects of both morphine and the narcotic partial agonist, pentazocine [224]. The effects of the α-adrenoceptor agonist, clonidine, are particularly interesting and for the moment deny adequate explanation. Thus, low doses of clonidine given ICV, like ICV NA, markedly antagonise the antinociceptive effects of both narcotic agonist and partial agonist analgesics (see *Figure 5.1*), confirming that in the periventricular tissues there are alpha-like adrenoceptors which play a positive modulating role in the transmission of nociceptive stimuli [66,68,225]; this is a conclusion supported by the observation that reductions in brain NA have a closer correlation with the antinociceptive effects of morphine than do changes in brain DA [226]. However, centrally-acting α-adrenoceptor agonists, such as xylazine, clonidine and St.-567, when given by conventional routes of injection, themselves exert marked antinociceptive activity of a high order and in a dose-related manner (see *Figure 5.2*). Thus, in addition to those alpha-like adrenoceptors affected by ICV injections of sympathomimetics, there are similar receptors at other sites of the cerebrospinal axis which have a diametrically opposite function. The exact role of alpha-like adrenoceptors in the CNS, particularly with respect to nociceptive sensitivity and the antinociceptive activity of centrally-acting analgesics, will differ from one site to another; a major disadvantage of ICV injections in rodents is the very wide distribution of materials in the brain following this route of administration,

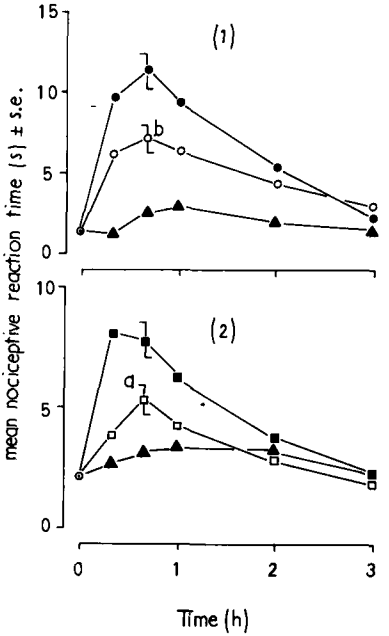


Figure 5.1. Antinociceptive activity of morphine (1) and pentazocine (2) in the mouse tail immersion test, alone and in combination with ICV-injected clonidine. Immersion temperature = 48° C. All drugs were given at time 0 h, when animals received: ICV-injected vehicle (saline) plus either morphine (3.0 mg/kg, subcutaneously, ●), or pentazocine (15 mg/kg, s.c., ■); others received ICV-injected clonidine (0.5 μg/animal) plus vehicle (s.c. saline, ▲), morphine (3.0 mg/kg, s.c., ○), or pentazocine (15 mg/kg, s.c., □). Mean group responses were compared by Student's t *test, and a* = P < *0.05 and b* = P < *0.01*

and better progress will be made by observing the effects of injections of material into more restricted CNS sites.

The involvement of catecholamines in tolerance to and dependence on opiate drugs has been studied extensively, through experiments designed to alter the normal functional states of catecholamines in the CNS. Both reserpine and tetrabenazine have been used in this connection, when both agents were reported as potentiating the severity of nalorphine-induced withdrawal effects in opiate-dependent animals [227,228]. Similarly, specific depletors of the catecholamines, such as α-MMT, or unspecific catecholamine blockers, such as the phenothiazines and butyrophenones, have each been shown to significantly reduce the severity of the abstinence syndrome [229–231]. Predictably, monoamine oxidase (MAO) inhibitors increase the severity of the abstinence syndrome in dogs [227], yet ameliorated the withdrawal effects in mice [228]. Furthermore, it

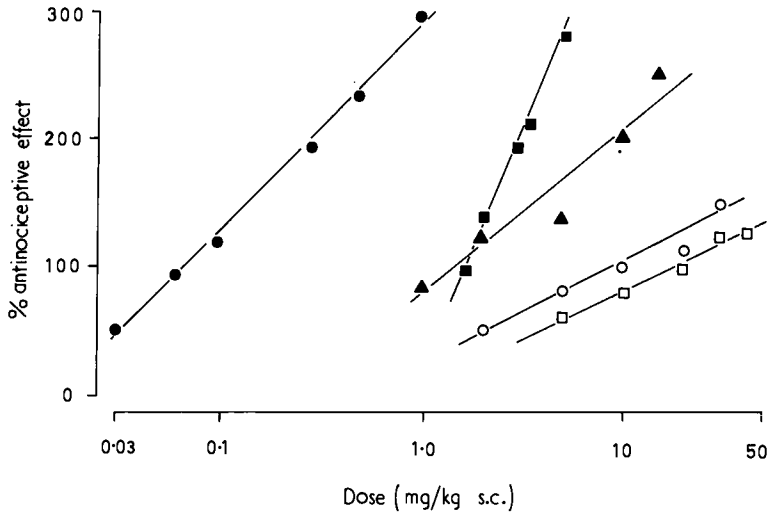


Figure 5.2. Comparison of log dose–response regression lines for the α-adrenoceptor agonists, clonidine (●), xylazine (▲) and St-567 (○) with those of the narcotic agonist morphine (■) and the narcotic partial agonist pentazocine (□), using the mouse tail immersion test. Immersion temperature = 48°C. The antinociceptive effect (%) was determined according to the method of Sewell [68,69]

has been shown that α-adrenoceptor antagonists cause a dose-related suppression of the morphine abstinence syndrome in rats [232], so that it is possible that α-adrenoceptor blockers will at least partially substitute for morphine under appropriate conditions in some species of animals.

Whilst there is now a very large body of evidence linking the catecholamines with opiate analgesia, and tolerance to, and dependence upon, the opiates, the vast array of experimental conditions, the numerous antinociceptive testing methods and the many species of animals used, all serve to provide a confusing picture of the catecholamine role; in addition, many effects have been produced using doses of opiate far in excess of those normally required to abolish nociceptive sensitivity. A number of facts stand out to provide a foundation for further work: the level of morphine analgesia is altered by changes in the levels or availability of the catecholamines, although NA appears to have different functions at different sites; stimulation of DA receptors is usually associated with a reduction in nociceptive sensitivity; studies with adrenoceptor blocking drugs suggest that beta-like receptors play no important role.

Whether there is a direct or indirect effect upon catecholamine transmission or whether morphine exerts presynaptic or postsynaptic (or both) effects is not known. It is impossivle, therefore, to integrate and explain the many apparently

contradictory observations that have been made until these effects are elucidated.

5-HYDROXYTRYPTAMINE AND NARCOTIC ANALGESICS

The presence of 5-hydroxytryptamine (5-HT) in the CNS of mammals was first demonstrated in 1953 [233] although its possible role as a neuronal transmitter in the brain was not considered until some years later [234,235]. Some workers have designated 5-HT as a mediator of pain [236–238], yet conversely, it can be shown that reductions in brain 5-HT levels following bilateral lesions in the subcallosal septal area, the medial forebrain bundle and midbrain tegmentum are associated with reductions in the nociceptive threshold in rats [239]. Similarly, lesions in the midbrain raphé nuclei of rats and cats cause marked falls in the 5-HT content of this area, with behavioural changes which may be interpreted as increases in sensitivity to painful stimuli [240]. Furthermore, when 5-HT is injected directly into the brains of mice (using the ICV injection technique), it possesses a transient but significant antinociceptive effect in the tail-flick test [114]. It is not known why ICV injections of 5-HT did not exert the same effect in the mouse hot-plate test or in the rat foot-pressure test [92,193], but in both species ICV injections of this amine consistently enhanced the antinociceptive effects of narcotic agonist and narcotic partial-agonist analgesics [114,193]. Clearly, the major part of presently available evidence supports the concept that endogenous 5-HT functions to inhibit the effects of nociceptive (painful) stimuli or that 5-HT is a positive modulator of antinociceptive activity.

Early experiments concerning the possible involvement of 5-HT with centrally-acting analgesics centred on attempts to measure steady-state brain levels of 5-HT during acute or chronic administration of morphine. A number of these reports suggested that single doses of morphine do not alter central 5-HT levels in the rabbit [241], dog or cat [242]. Also, 5-HT synthesis appears to be unaffected by single doses of morphine in mice [243,244], although slight increases in 5-HT turnover have been observed in studies using the rat [245].

In contrast, there have been other reports that morphine does alter brain levels of 5-HT, with sometimes an increase [246] and sometimes a decrease [247,248]; this confusion is tempered slightly by the knowledge that the effects of morphine on brain levels of DA and NA, as well as of 5-HT, are modified significantly by the dose of opiate employed [249]. Thus, there appears to be no simple monophasic type of relationship between morphine-induced changes in brain biogenic amine levels and antinociceptive activity.

The picture is similar in sub-acute and chronic studies using morphine, but the majority of investigators have found no direct effect of morphine on the levels of 5-HT in the brain of several species [129,242,244,250–253].

In other studies, there have been attempts to relate the pharmacological effects of morphine to induced changes in the turnover of 5-HT and its metabolites in the brain, the most frequently used manoeuvre involving an assessment of whole-brain 5-HT levels after inhibition of its conversion by monoamine oxidase (MAO) to 5-hydroxyindole acetic acid (5-HIAA), using a MAO inhibitor (MAOI). Thus, increases in 5-HT turnover have been observed during opiate tolerance [243,244,254,255], yet this was not observed in chronic morphine treatment [256]. In 1971, Cheney, Goldstein, Algeri and Costa measured 5-HT turnover by studying the conversion of radioactive tryptophan into 5-HT and they found no increase in turnover in this pathway in either tolerant or dependent animals. Different interpretations have been put on results from studies of the accumulation of 5-HIAA in probenecid-treated animals, when the efflux of this acid metabolite from the brain is arrested [257]. Under these circumstances, morphine induced significant increases in rat brain indole turnover [257], an observation subsequently confirmed in other laboratories [258,259]; yet, this is an effect apparently not induced by other narcotics (for example, pethidine, methadone) or a narcotic partial agonist (pentazocine).

A variety of other pharmacological and biochemical techniques have been used to manipulate brain 5-HT levels and turnover, with a view to relating the pharmacological properties of morphine to indole function. Thus, elevation of brain 5-HT after MAOI administration causes an enhancement of the effects of both morphine [260] and pethidine [261] remembering that this procedure (MAO inhibition) increases brain levels also of both DA and NA. Treatment with an MAOI also potentiates the effects of morphine in tolerant and non-tolerant mice, the degree of augmentation being similar in both types of animal [262].

5-Hydroxytryptophan (5-HTP), the immediate amino acid precursor of 5-HT, not only possesses antinociceptive activity when administered alone [176,263], but also increases the antinociceptive activity of morphine [171,176,237] in intact animals and partially restores the antinociceptive activity in animals rendered insensitive to morphine by pretreatment with reserpine [171,264]. Likewise, injections of the amine (5-HT) itself potentiate the antinociceptive effects of certain analgesics; thus, in both rats and mice, ICV injections of 5-HT potentiate the effects of morphine [114,193,265], of other narcotic agonist analgesics of opiate and non-opiate structure, and also of the narcotic partial agonists. On each occasion, the ICV injection of NA had directly opposite effects; that is, ICV NA markedly attenuated the antinociceptive activity of these analgesics in both species [66,68,219,225]. These effects of ICV 5-HT and NA are not elicited when considerably larger doses of amine are administered by subcutaneous injection, strongly suggesting that the interactions observed between the amines and the various analgesics are discharged at a central, probably periventricular, level. Pretreatment of rats and mice with reserpine markedly reduces the antinocicep-

tive effects of both morphine (narcotic agonist) and pentazocine (narcotic partial agonist), and the antinociceptive effects of these analgesics are restored by the ICV injection of 5-HT, but remain unaffected by ICV NA [68]. From these findings, it has been proposed that the acute antinociceptive effects of narcotic agonist and partial agonist analgesics are determined by a dynamic balance within the brain between the two amines, 5-HT and NA [66,225]. A disturbance in the function of either amine may cause a change in the antinociceptive activity of these analgesics, and so a depletion of tissue stores of NA or a blockade of post-synaptic NA receptors may produce qualitatively a similar effect as increasing the activity of 5-HT in the brain. Conversely, the blockade of post-synaptic 5-HT receptors (for example, with the antagonist cyproheptadine) may abolish the antinociceptive activity of morphine in a manner qualitatively similar to the ICV administration of the amine NA. Work with the other catecholamine DA has been only spasmodic but, like NA, it has been designated a negative modulator of narcotic analgesia [266].

The above findings and proposals have been substantially confirmed by studies involving the depression of available 5-HT levels in the brain, either through the administration of blocking agents or through lesions placed in specific 5-HT neurones. Thus, the administration of *p*-chlorophenylalanine (*p*-CPA), an agent which reduces tissue 5-HT levels through inhibition of the enzyme tryptophan hydroxylase (EC 1.14.16.4) [267], causes the development of a hyper-nociceptive state coincident with the depletion of tissue 5-HT stores [238,268]. At the same time, the antinociceptive effects of morphine are substantially reduced [161,192,269] but can be restored by the addition of the precursor 5-HTP [270]. Consistent with these observations, the selective depletor of tissue 5-HT, *p*-chloroamphetamine, and the 5-HT receptor antagonist, cyproheptadine, antagonise the analgesic effects of both narcotic and non-narcotic drugs in man [250]. More recently, 5,6-dihydrotryptamine, a new long-lasting selective depletor of tissue 5-HT, when injected centrally into rats so that the depletion was restricted to brain stores, produced a marked and persistent antagonism of the antinociceptive effects of morphine [271]. These observations taken together further substantiate the suggestion that 5-HT exerts a negative modulatory role in the transmission of nociceptive stimuli through the cerebrospinal axis (or exerts a positive mediatory role in narcotic analgesia).

Electrolytic lesions to the nucleus raphé dorsalis cause a marked decrease in forebrain 5-HT levels which results in an antagonism of morphine analgesia [272,273], but not of methadone or pethidine analgesia [274]. This led the authors to suggest that morphine may be atypical (amongst opiate analgesics) in its reliance upon intact 5-HT pathways. There have been other reports that methadone [146] and pethidine [258,259] behave differently from morphine, as they do not increase rat brain 5-HT turnover following their acute administra-

tion. Yet in mice, methadone increases central 5-HT turnover [275]. More recently, it has been demonstrated that fluoxetine [276], a specific and potent inhibitor of 5-HT re-uptake into pre-synaptic stores [277,278], at a dose which alone exerts no antinociceptive activity, potentiates the effects of morphine but not of methadone or pethidine in rats. This again suggests that morphine is dissimilar from other agents in the opiate series. However, whereas morphine is only a weak inhibitor of the re-uptake of 5-HT into neurones [279,280], both pethidine [281] and methadone [280,282] are relatively potent uptake blockers. Although this property in itself would be expected to raise 5-HT turnover in the brain, it may simultaneously result in less reliance of either pethidine or methadone on intact 5-HT pathways, since relatively low levels of tissue 5-HT (as might remain after lesioning techniques) would suffice to maintain the function of 5-HT neurones in the presence of a potent uptake inhibitor.

It has been shown that the ability of reserpine to antagonise the effects of morphine is greatly enhanced in animals already made tolerant to an opiate [191]. Although the degree of 5-HT depletion by reserpine is similar in tolerant and non-tolerant animals, tolerant animals recover from the CNS depressant effects of reserpine more quickly. As this recovery correlates with a more rapid repletion of 5-HT stores, 5-HT may be involved in the development of tolerance. This view is largely substantiated by the finding that inhibition of 5-HT synthesis by *p*-CPA partially prevented the development of tolerance to, and also the physical dependence upon, morphine [244,254]. Likewise, selective depletion of 5-HT stores using 5,6-dihydroxytryptamine also reduced the degree of tolerance and dependence in animals treated chronically with morphine [283]. In earlier work from the same laboratory, Loh, Shen and Way [243] showed that the protein synthesis inhibitor, cycloheximide, when administered concurrently with daily injections of morphine, inhibited the development of tolerance to, and the physical dependence on, morphine; other workers have confirmed this finding using the protein synthesis inhibitor, actinomycin D [284]. From these data, it has been proposed that the synthesis of protein concerned in the onset of tolerance has been inhibited by the cycloheximide or actinomycin. It is possible that the synthesis of a protein with a more rapid turnover rate than that of a receptor is prevented by the inhibitors, and this protein may be associated with the synthesis of 5-HT. Recently, Ho, Brase, Loh and Way [285] showed that the rate at which tolerance to a narcotic agonist analgesic developed in rats and mice was augmented by daily administration of the 5-HT amino acid precursor, L-tryptophan. Furthermore, this augmentation was blocked by the simultaneous administration of *p*-CPA which prevents the conversion of tryptophan to 5-hydroxytryptophan by the enzyme tryptophan hydroxylase (EC 1.14.16.4). This evidence supports the view that alterations in the synthesis of 5-HT occur in, and may be responsible for the development of, tolerance to narcotic agonist analgesics.

By comparison with the evidence implicating other amines, the data available for 5-HT are consistent. Impairment of central 5-HT function, for example, is associated with a loss of analgesic activity whilst enhancement of 5-HT pathways is accompanied by increases in the antinociceptive activity of morphine and probably other narcotic agonist and partial agonist analgesics. There is also evidence consistently implicating some derangement of 5-HT function in the development of tolerance and physical dependence. It has been suggested that morphine may influence the cortical cholinergic system through neurones originating in the raphé nucleus [286]. Others have indicated that dopaminergic pathways may be subject to tryptaminergic regulation in the expression of naloxone-precipitated withdrawal symptoms in mice [287]. The hypotheses are attractive because they link two or more transmitter systems. The simultaneous and interrelated changes in the function of several amines which this approach assumes are largely supported by the mass of complex and often conflicting evidence which is currently available.

HISTAMINE AND NARCOTIC ANALGESICS

Considerable interest has centred recently on the possible transmitter role of histamine [288–290], an amine known to be released both peripherally [291] and centrally [292] by morphine. A central transmitter role for histamine has been considered and so there is interest in its possible involvement in opiate analgesia. Also, it has been demonstrated that some antihistamines (such as mepyramine which is an antagonist of the H_1 receptors) antagonise some of the central effects of morphine [293], including antinociceptive activity in rabbits [294]. However, these effects may be due to atropine-like or even anti-5-HT activity.

Endogenous histamine concentrations in the hypothalamus, brain stem and cerebral cortex are not significantly changed in rats after acute injections of morphine [295]. However, after chronic morphine treatment, decreases in histamine levels have been observed in all three brain areas, but particularly in the hypothalamus which normally has a high concentration and rapid turnover of the amine [296]. This suggests therefore that reductions in histamine levels are associated with, and may even be the cause of, the development of tolerance. However, mepyramine (a H_1 receptor antagonist), when administered chronically with morphine, inhibits the development of dependence but not the induction of tolerance in mice [297]. An increase in central levels of histamine 24 h after the administration of mepyramine was also noticed in the same study. Although brain levels of histamine in mice are similar in tolerant and non-tolerant mice [298], this does not necessarily mean that chronic treatment with morphine has no effect on central histamine function, since changes in the rate

of turnover of the amine may occur without concomitant changes in tissue levels [299]. Thus, the ability of mepyramine to prevent the development of dependence upon morphine can be explained in terms of H_1 receptor antagonism or modification of the release and/or turnover of brain histamine.

Since morphine releases histamine from central stores [292] and chronic administration of histamine alters brain catecholamine levels [300], it has also been proposed that opiate-induced changes in central concentrations or tissue levels of other biogenic amines may be initiated by decreases in brain histamine [295]. Whilst a role for brain histamine has yet to be determined in the antinociceptive effects of the narcotic analgesics, this amine may interact with one or more other amines to subserve the antinociceptive, tolerance and physical dependence effects of morphine-like agents.

RECENT DEVELOPMENTS

Other naturally occurring substances, such as the cyclic nucleotides and prostaglandins, have been associated recently with the narcotic agonist analgesics, when it was suggested that analgesia may be induced by inhibiting a prostaglandin E_1-stimulated adenylate cyclase mechanism [301]. Another novel action of the opiates was described in a neuroblastoma–glioma hybrid cell line (in culture), when it was found that both morphine and levorphanol (both narcotic agonist analgesics) stimulated cyclic GMP formation, and simultaneously depressed concentrations of cyclic AMP [302]. The stimulation of cyclic GMP synthesis might be responsible for the opiate-induced reductions of cyclic AMP levels and also for their inhibition of prostaglandin-stimulated cyclic AMP production, since both nucleotides are reciprocally related in a number of biological studies [303]. Additional evidence has been presented to suggest that both morphine and the narcotic antagonist, naloxone, stimulate prostaglandin biosynthesis [304]. However, dependence upon opiates may be induced by the agonist actions of these agents. If the agonist action of morphine is mediated through the inhibition of adenylate cyclase in morphine-sensitive neurones, then dependence might occur as a consequence of homeostatic enhancement of adenylate cyclase activity which is then physiologically excessive during abstinence [304].

Recently, the endogenous morphine receptor ligand, enkephalin, has been isolated [305], identified chemically [306], and found to possess antinociceptive activity in mice after ICV injection [307]. The interactions of the amines, enkephalin, and the cyclic nucleotides remain to be determined, and the scope for future studies of the mechanisms of action of the opiate analgesics becomes much wider as a result of these more recent studies.

CONCLUSIONS

There now exists a substantial body of evidence linking the pharmacological actions of narcotic agonist and partial agonist analgesics with each of the CNS neurotransmitters: ACh, DA, NA and 5-HT. The actions of these analgesics are also linked with histamine, but until a more definite transmitter function has been ascribed to histamine, our acceptance of its involvement in the transmission of nociceptive stimuli in the cerebrospinal axis (and/or the pharmacological actions of narcotic analgesics) must be guarded. Perhaps the availability of the H_2-receptor antagonist, cimetidine (or hopefully cimetidine analogues with access to the brain) will resolve that particular problem.

A fuller appreciation of the interactions of morphine-like agents with the biogenic amines is important for a number of reasons. First, it may help to identify the components of the spectrum of sub-cellular actions that morphine exerts and so enable new analgesics to be obtained with more specific properties. Secondly, and related to the first, it may prove possible to identify those components which are involved in tolerance and physical dependence. On present evidence, rather than being able to separate analgesia from tolerance and dependence, the amine studies tend to confirm that these three properties are firmly associated with each other. Both tolerance and physical dependence remain the inevitable long-term consequence of narcotic agonist activity, and all three phenomena may ultimately be explained on the same aminergic basis. Studies with ICV-injected agents have revealed that centrally-acting analgesics of a fundamentally different type do exist (for example, the α-adrenoceptor agonists such as clonidine and xylazine) and they exert qualitatively different interactions with the biogenic amines. The differences which exist between the morphine-like and clonidine-like analgesics can be demonstrated by the acute ICV injection of the amines, NA and 5-HT, in rodents, and this technique promises to be a more rapid and economic method of demonstrating morphine-like or non-morphine-like properties in a new analgesic than resorting to long-term studies in primates.

Finally, the demonstration that narcotic analgesics interact with brain amines suggests that these analgesics may interact with other psychotropic drugs, like the MAO inhibitors, the tricyclic antidepressants and the neuroleptics.

Some of these interactions may be potentially harmful (for example, the potentiation of opiates by MAO inhibitors); some interactions may help reduce the dose of, or frequency of dosing with, opiates so that there may be useful retardation of the development of tolerance and dependence. Other interactions may be less predictable but nevertheless important; thus, both reserpine and chlorpromazine are neuroleptics and exert anti-psychotic activity in man, but whereas chlorpromazine may induce a useful potentiation of morphine analgesia in man (a property only partially explained on the basis of impaired hepatic

metabolism of morphine), reserpine exerts a substantial attenuation of the analgesic actions of morphine in both animals and man.

Although the greater part of the data described in this review has been obtained in experimental animals, the nociceptive techniques used possess a significant predictive value for subsequent studies in man. Thus, the continuing study of brain amines in relation to pain and analgesia provides a sound foundation for the detection and evaluation of new and safer analgesics.

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6 Flavonoids: Some Physiological and Nutritional Considerations

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INTRODUCTION

The term flavonoid as currently used refers to a large group of chemical compounds characterised by a C_6–C_3–C_6 carbon skeleton where the C_6 components are aromatic rings. The classification, structure, biosynthesis and distribution of the flavonoids have been extensively documented in recent years [1–5]. There is evidence that certain flavonoids have pharmacological, and possibly nutritional, properties; these have been variously referred to as 'vitamin P' and 'bioflavonoids' (*Figure 6.1*).

Flavonoids – along with other 'secondary' plant products – have long been

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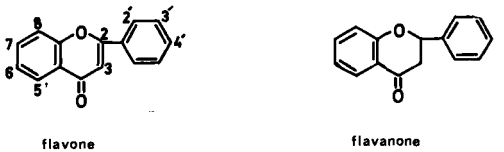

flavone flavanone

Figure 6.1. Flavonoids shown to be of nutritional significance are, for the most part, derivatives of either flavone or flavanone, e.g. quercetin (3,3,4,5,7-pentahydroxyflavone), rutin (quercetin-3-rutinoside), naringenin (4,5,7-trihydroxyflavanone), hesperetin (3,5,7-trihydroxy-4-methoxyflavanone) and hesperidin (hesperetin-7-rutinoside)

regarded as metabolically inert components representing, in biochemical terms, a non-usable energy potential and, because of this, having no obvious definable function in primary metabolism. This viewpoint has been challenged in recent years on evolutionary grounds that no biochemical mechanism is likely to survive unless it leads to a product which confers some advantage [6]. The outlines of the biochemical evolution of flavonoids are now beginning to emerge [6]. They are of widespread distribution in the plant kingdom being virtually ubiquitous in Angiosperms and existing also in more primitive groups such as green algae (*Charophycaeae*), *Bryophyta* and *Hepaticae.* Nearly two thousand naturally-occurring flavonoid substances have been recorded in the literature [5].

Higher animals have, therefore, evolved in an environment in which their feeding habits have, of necessity, exposed them to a wide range of flavonoid material as 'secondary' components of foodstuffs. In contemporary terms, a person's daily intake of flavonoid material may be considerable; for example, some forty flavonoids have been characterised from citrus sources alone [2,4,7]. It is, therefore, not inconceivable that this evolutionary exposure to dietary flavonoids has elicited physiological and biochemical responses in higher animals. However, despite a considerable body of research during the last twenty years, the question of the pharmacological – and to a greater extent, the nutritional – significance of flavonoids remains largely unresolved.

Two points of general interest contribute to the complexity of the situation. The first is that ingested flavonoids are metabolised in higher animals to give a wide range of apparently non-toxic metabolites, many of which may be of physiological significance; some of these metabolites are produced in organs after absorption of the parent material, whereas others are formed previous to absorption by the intestinal flora – and in particular by bacterial hydroxylation [2,3]. A second complicating feature is that flavonoids possess considerable structural variety and metabolic versatility and attempts to confine their supposed pharmacological and nutritional attributes to (a) their ability to chelate with metals and (b) a direct antioxidant activity associated with the free hydroxyl groups at the 3′,4′ positions probably represent an oversimplification.

Flavonoids, therefore, possess considerable potential for intervention at the physiological and biochemical level, the areas of potential influence are diverse and numerous and a satisfactory definition of their role in human physiology is correspondingly difficult.

HISTORICAL BACKGROUND

Studies by Hungarian workers in the mid 'thirties indicated that a number of vegetables and fruits (notably citrus ones) contained substances capable of correcting certain abnormalities associated with scurvy. Guinea-pigs deprived of dietary vitamin C (L-xyloascorbic acid, ascorbic acid) rapidly develop scurvy, death ensuing in about 25–30 days. The early publications from Hungary indicated that the survival period of scorbutic guinea-pigs was prolonged by 'citrin', a flavonoid preparation, and that capillary fragility – a condition characteristic of ascorbic acid deficiency – was corrected by extracts from *Citrus limon* and *Capsicum annuum* [8,9]. The new factor was regarded as separate from vitamin C and was designated 'vitamin P' [10]. Later work, however, both by the original investigators and by others, failed to confirm the effect on guinea-pig survival time [11–13]; the earlier erroneous results were probably obtained because of the presence of traces of ascorbic acid in the flavonoid preparations. Bentsath and Szent-Gyorgyi nevertheless suggested that "vitamin P requires for its activity the presence of traces of ascorbic acid" and that "in the entire absence of ascorbic acid, 'vitamin P' is inactive" [14].

In 1949 Scarborough and Bacharach lucidly summarised the work done between 1935 and 1949 [15]. At that point the relationship between flavonoids and ascorbic acid was still awaiting resolution; it was not known whether flavonoids exerted a direct effect upon tissue metabolism (and, in particular, upon capillary fragility) or whether the effects attributable to flavonoids resulted from a synergistic or substitutive relationship with ascorbic acid. Scarborough and Bacharach dismissed the possibility that flavonoids could prolong the life-span of scorbutic guinea-pigs by a substitutive role in lieu of ascorbic acid and their main discussion centred on the possible role of flavonoids in enhancing capillary resistance (they used the term capillary resistance to embrace both capillary permeability – the flow of fluid across the capillary wall and its pathogenic consequence, oedema – and capillary fragility – the passage of erythrocytes across a ruptured capillary wall, with consequential purpurea). Lowered capillary resistance had long been regarded as a characteristic of scurvy, i.e. of ascorbic acid deficiency; it could equally conceivably have been a consequence of flavonoid deficiency as, in general, an ascorbic acid-deficient diet would also be low in flavonoids. Scarborough and Bacharach showed that it was difficult to correlate

capillary resistance with ascorbic acid status and they quoted with approval an earlier statement that "guinea-pigs placed on a scorbutic diet supplemented with adequate amounts of ascorbic acid show a decline in capillary strength ... restorable by administration of vitamin P" [16]. Thus, it appears that the consensus of opinion in 1950 was that flavonoids were of physiological significance, that their main, and possibly only, sphere of influence was on capillary resistance, and that their activity was probably independent of ascorbic acid. The contradictory results reported by some of the earlier investigators probably stemmed from (i) the difficulty of obtaining a completely flavonoid-free diet, and (ii) the failure to use ascorbic acid-free preparations of natural flavonoids.

POST-1950 STUDIES

The succeeding quarter of a century since the Scarborough and Bacharach review has witnessed a considerable proliferation in the suggested physiological involvements of flavonoids both in terms of clinical reports and of experimental studies with small animals. Bohm in his monograph has listed some forty definable influences of flavonoids in man; these include therapeutic, pharmacological and nutritional relationships [17]. In many areas, the evidence for flavonoid intervention is weak and 'confirmatory' studies have frequently given contradictory results. Nevertheless, there are a number of clearly defined areas where it appears that flavonoids are able to modify a physiological situation. Some of these flavonoid-induced modifications are probably, in the final analysis, attributable to changes in capillary resistance; an obvious candidate for inclusion in this category is the ability of flavonoids to depress various types of inflammatory reaction. Gabor has described the beneficial effects of flavonoids in a number of model inflammatory reactions induced by agents such as cotton wool pellets, mustard oil and UV radiation [18]. Other pharmacodynamic effects of flavonoids, however, are not so easily attributable to correction of a basic lesion such as altered capillary resistance. Recent studies have indicated that flavonoids may influence metabolism in such apparently unrelated areas as hepatic detoxication [19], thymus function [20], anaphylactic shock [21], and lipid utilization [22,23].

Despite the emergence of these 'new' areas of activity, the main tendency is still to regard flavonoids as primarily influencing capillary resistance; this is certainly the area in which flavonoid preparations have attained greatest therapeutic significance. The biochemical *modus operandi* of flavonoids – both in terms of capillary resistance and against a more general background of physiological and nutritional significance – remains unrevealed. Suggested mechanisms for flavonoid activity include enzyme inhibition (particularly of hyaluronidase), prolongation of adrenaline action by inhibition of *O*-methyltransferase (a key enzyme in its

degradation), stimulation of the pituitary–adrenal axis (of interest here is the inhibition by epicatechin of the adrenal hypertrophy of scurvy [13]), and potentiation and/or 'sparing' of ascorbic acid [3,24].

A number of factors militate against a rapid resolution of the problem. There is no simple biochemical test for assessing flavonoid potency or for detecting any possible deficiency. Most 'quantitative' studies are based on measurements of capillary permeability or capillary fragility (e.g. refs. 25,26). In the case of human studies, it is impossible to use control groups because of the virtually ubiquitous distribution of flavonoids in most foodstuffs – a fact used by Szent-Gyorgyi to explain why conditions of flavonoid deficiency are unlikely to emerge in countries where the intake of citrus material is high [27]. The situation is further complicated by the diversity of structure and biological potency amongst the flavonoids, by the wide range of flavonoid metabolites of largely unknown metabolic significance, and by the apparent inter-relationships between flavonoids and specific nutrients, and in particular ascorbic acid.

FLAVONOIDS AND ASCORBIC ACID

The first indications of the possible nutritional significance of flavonoids emerged during studies on ascorbic acid [8,9]. Since then, probably the most widely used 'model system' for examining the biochemical significance of flavonoids in animals has been their supposed relationship to ascorbic acid. There is widespread evidence that flavonoids may influence the metabolism of ascorbic acid but the exact nature and significance of the relationship are not known. It is of interest to note that a number of the less clearly definable areas where flavonoids are believed to have metabolic involvements are areas where ascorbic acid too is believed by some to be of physiological significance. There are claims that flavonoids, like ascorbic acid, are hypocholesterolaemic and hepatodetoxicogenic [17,19,22,23,28] and a recent report of rutoside-induced modifications in the basement membrane brings to mind the observation that the basement membrane contains a collagen-like peptide sequence for whose synthesis ascorbic acid is presumably an obligatory factor [29–31]. Attempts to characterise the flavonoid–ascorbid acid relationship have, in general, centred on three main areas:

1. The influence of ascorbic acid on the 'capillary resistance' effect of flavonoids.
2. The influence of flavonoids on growth and survival *vis-a-vis* ascorbic acid status.
3. The influence of flavonoids on the accumulation and metabolism of tissue ascorbic acid.

This is an arbitrary and somewhat artificial system of categorisation and the three possible areas of interaction are by no means mutually exclusive. Thus, any measurable effect on growth (2) could theoretically be a consequence of a changed metabolism or availability of ascorbic acid (3).

ASCORBIC ACID, FLAVONOIDS AND CAPILLARY RESISTANCE

Scarborough and Bacharach implied that impaired capillary resistance was more to be attributed to lack of flavonoids rather than to ascorbic acid deficiency. Since then the exact relationship between flavonoids and ascorbic acid with respect to capillary resistance has remained a matter of some dispute. French workers [32,33] indicated that changes in capillary fragility in guinea-pigs given a flavonoid-free diet were not preventable by ascorbic acid – a finding not in accord with a later report that no factor other than ascorbic acid was necessary to prevent capillary fragility [34]. Ambrose and De Eds reported that flavonoids (rutin, quercetin) decreased the haemorrhages that characterised hypovitaminosis C in guinea-pigs [35] and more recently Robbins reported that rutin, hesperidin and naringin enhanced the therapeutic effect of ascorbic acid on the capillary resistance and blood cell aggregation changes which appeared in ascorbic acid-deficient guinea-pigs [36]. It has been suggested that the capillary resistance effect of flavonoids is mediated by their inhibition of hyaluronidase, hyperactivity of which may reduce the capillary intercellular cement; Beiler and Martin have claimed that flavonoids such as hesperidin inhibit hyaluronidase *in vitro* only in the presence of ascorbic acid [37] – a synergistic relationship which they later demonstrated *in vivo* [38]. Flavonoids possess a therapeutic value in correcting capillary deficiencies; this does not mean that they are necessarily an obligatory nutrient for the maintenance of normal capillary resistance. As capillary fragility is a feature of ascorbic acid deficiency, the question of a possible ascorbic acid–flavonoid synergism in this context is not one that can easily be resolved by animal experimentation.

ASCORBIC ACID, FLAVONOIDS AND THE GROWTH OF EXPERIMENTAL ANIMALS

One consequence of the influence of certain flavonoids on capillary resistance and their possible involvement as an obligatory factor for the maintenance of capillary integrity was their designation as a specific nutritional factor – and not merely as an adjunct to ascorbic acid action. They were collectively termed 'vitamin P' [27] or bioflavonoids. The term 'vitamin P' continued in use until 1950 when the Joint Committee on Nomenclature of the American Society of Biological Chemists and the American Institute of Nutrition recommended its

discontinuation; this was followed in 1968 by the possibly more debatable statement by the U.S. Food and Drug Administration that flavonoids possessed no efficacy in man 'for any condition' [39].

Certainly it would be difficult to adduce evidence that flavonoids should be regarded as essential nutritional factors in the generally accepted sense of the definition. A *sine qua non* of an obligatory nutrient is that, in its absence, normal development is impaired (both qualitatively and quantitatively) and death occurs; the essentialness of a nutrient is easily determined using experimental animals and an appropriate synthetic diet. There is little evidence that flavonoids satisfy these criteria, and there are many records of guinea-pigs and of rats surviving for considerable periods on flavonoid-free synthetic diets. Fabianek, Neumann and Lavollay obtained good growth and health in guinea-pigs given a flavonoid-free diet over a period of 13 months [34].

One is justified, in nutritional terms, in distinguishing between an 'essential nutrient' and a 'growth factor'. All essential nutrients are growth factors but not all growth factors are essential nutrients: in the absence of a growth factor, maximum growth may not be achieved but death does not ensue. In a sense, all utilisable nutrient materials are growth factors; as generally used, however, the term refers to those non-obligatory factors which appear to stimulate the growth of organisms receiving a satisfactory diet in terms of general nutrient requirements. Theoretically, growth factors may operate in one or more of three ways: substitutively by 'taking over' from an essential nutrient in one or more of its metabolic roles, synergistically by enhancing the growth-maintenance capacity of a nutrient, and complementarily where the growth factor is a nutrient which the body can itself synthesise only to a limited extent.

There is evidence that in certain circumstances flavonoids have growth factor characteristics. Perhaps the most striking example of the influence of flavonoids on growth on an otherwise complete diet was the 2–3-fold increase in the growth rate of the house cricket (*Acheta domesticus*) produced by rutin, hesperidin and hesperidin methyl chalcone [40]. Of equal interest are indications of a possible ascorbic acid–flavonoid interaction in terms of growth. Despite the occasional claim that certain flavonoids are able to prolong the life of scorbutic guinea-pigs [41] the bulk of the evidence is that they are unable, completely or partially, to substitute for ascorbic acid in the prevention of scurvy. However, survival experiments of this type are unsatisfactory because they take no account of the inanition which is characteristic of the 3–4 days immediately preceding death, and the possibility that ascorbic acid has, in addition to its accepted biochemical role, a specific 'appetite' effect. A compound which can replace ascorbic acid biochemically will not necessarily prolong the survival time if the guinea-pigs are dying of inanition. To overcome this difficulty, a liquid flavonoid-free scorbutogenic diet for manual feeding has been formulated [42] and used to

obtain a more complete characterisation of the final stages of the disease. A recent study using this technique has indicated that a flavonoid-rich preparation of orange peel, known to influence tissue levels of ascorbic acid (see next section), had no influence on the survival time of acutely scorbutic guinea-pigs [43]. One is forced to conclude that flavonoids are unable to act in a complete substitutive capacity for ascorbic acid.

These essentially negative results are in contrast with the apparent ability of flavonoids to modify the course of chronic scurvy (hypovitaminosis C) – the condition produced when guinea-pigs receive 'subminimal' quantities of ascorbic acid over a long period; in these circumstances, both rutin and quercetin reduce the scorbutic symptoms and prolong the survival time [35]. Similar effects of flavonoids on growth at suboptimal ascorbic acid intakes have been recorded. This effect has been shown to occur with hesperidin [44], quercetin and epicatechin [22], with a flavonoid-rich blackcurrant juice concentrate [45] and with an extract of orange peel [46]. In the latter study, guinea-pigs received a maintenance dose of 0.1 mg of ascorbic acid per 100 g body weight daily, which was insufficient to produce any gain in body weight. Administration of 5 mg of an ascorbic acid-free extract of orange peel daily to a test group produced a striking increase in body weight. A similar effect was produced by 5 mg hesperidin daily; hesperidin is one of the main flavonoids in orange peel [47,48]. This 'growth factor' effect of flavonoids was only apparent when the ascorbic acid intake was limiting with respect to growth; neither hesperidin nor the orange peel extract had any effect on the growth of ascorbic acid-sufficient guinea-pigs [49].

DIETARY FLAVONOIDS AND TISSUE ASCORBIC ACID

Studies with guinea-pigs

A possible explanation of the 'growth factor' effect described above is that flavonoids 'spare' ascorbic acid either by taking over one or more of its physiological functions or by protecting it against intestinal or tissue breakdown. This would release additional ascorbic acid for growth support. This is an attractive hypothesis supported by the finding that flavonoids administered to guinea-pigs on a restricted ascorbic acid intake almost invariably induce elevated tissue ascorbic acid levels. This has been demonstrated with rutin (quercetin-3-rutinoside) [50, 51], catechin [52], hesperidin [46], trihydroxyethyl rutoside [53] and with flavonoid-rich extracts of blackcurrant [45], orange peel [46] and green tea [54]. Charley's review of the field in the 1960's contained reference to much generally-inaccessible material including Russian studies where elevated tissue ascorbic acid levels in guinea-pigs were demonstrated after the administration of "hesperidin, quercetin, rutin, eridictyol, catechin, tea leaf extracts and grape

tannins" [7]. Of relevance in this context is the report by Crampton and Lloyd that, in hypovitaminotic C guinea-pigs, rutin increased the apparent biological value of ascorbic acid by over 50% as measured by the odontoblast cell technique [55]. It is, however, debatable whether increases in ascorbic acid of the order obtained in some of these studies would be quantitatively sufficient to produce the type of growth response obtained [46]. The majority of these studies are characterised by two features:

1. the elevation of tissue ascorbic acid is most readily demonstrated when the ascorbic acid intake is marginal; reports of an effect in ascorbic acid-sufficient animals are rare;
2. the flavonoid-induced elevation of tissue ascorbic acid is most marked in, and is sometimes limited to, the adrenal glands.

Human studies

Fewer studies have been made with man and they have, of necessity, been confined to the influence of dietary flavonoid supplements on blood and urinary ascorbic acid. A weakness of such studies is that the diet of most subjects contains a substantial amount of flavonoid material – even when obvious sources such as citrus fruits are excluded from the diet. This makes difficult the inclusion of a control group and also reduces the significance of additional flavonoid supplements. Nevertheless, there is some evidence that flavonoids may modify the absorption–retention–stability of ascorbic acid in man.

Such evidence as is available suggests that increased flavonoid ingestion increases the urinary output of ascorbic acid in subjects on a controlled acid intake [49,56,57]. In males receiving a controlled intake of 75 mg of ascorbic acid daily, both orange flavonoids and rutin increased the urinary ascorbic acid output [57]. These results were confirmed by other workers using a method designed to eliminate from the urine interfering substances which would otherwise give a falsely high value for ascorbic acid [49,58]; the mean urinary ascorbic acid excretion (mg/h) over the first 6 h following a dose of 80 mg of ascorbic acid was 3.1 in the case of subjects given 'synthetic' ascorbic acid and 6.8 for those given the acid in flavonoid-containing orange juice (the difference between the two groups being of a high order of significance ($P < 0{,}001$) [49]). In a parallel set of experiments, 'orange juice ascorbic acid' produced higher leucocyte ascorbic acid concentrations than a corresponding intake of a flavonoid-free ascorbic acid preparation [59]. Results of this type are consistent with an increased absorption of ascorbic acid in the presence of flavonoid – although a report that flavonoids may modify the ascorbic acid : dehydroascorbic acid ratio in plasma implies that mediation at the tissue level cannot be entirely discounted [60].

Whether the same basic mechanism is accountable for these flavonoid-induced ascorbic acid changes in both the guinea-pig and man is not known. Certainly there can be little doubt that flavonoids may, under specific conditions, influence the tissue concentrations of ascorbic acid in the guinea-pig and probably in man, too. A number of distinct, but not mutually exclusive, explanations have been proferred to account for the apparent enhancement by flavonoids of ascorbic acid status in guinea-pigs. Four of these merit some discussion.

Flavonoid enhancement of ascorbic acid absorption. Raised tissue concentrations of ascorbic acid could be the result of enhanced gastro-intestinal absorption of the acid in the presence of flavonoid material. Theoretically, this could imply a direct involvement of flavonoid in the ascorbic acid-absorption process or it could be an indirect consequence of the ascorbic acid-stabilizing influence of flavonoid (see below). Degradative oxidation of ascorbic acid resulting in its conversion to substances lacking vitamin C activity would be expected to occur at the pH and temperature of the small intestine [61]; by stabilising ascorbic acid, the flavonoid would increase its exposure time to the absorption processes. A system of this type would become less efficient as the ascorbic acid 'absorption ceiling' was approached; this would be in agreement with the finding that flavonoid enhancement of tissue ascorbic acid is most marked at low intakes of the vitamin. A possible weakness of the 'enhanced absorption' theory in both its 'direct' and 'indirect' forms is that one would expect the increased ascorbic acid absorption to be reflected by increases in the acid concentration in most tissues (with the possible exception of the brain, which is somewhat resistant to dietary induction of changes in its ascorbic acid status [62]). In most cases, it is the adrenal ascorbic acid which is most significantly elevated, sometimes with no change at all in its concentration in other organs [46,50,51]. This indicates that the flavonoid–ascorbic acid interaction is a feature of specific organs such as the adrenal – or at least, that it is not solely a reflection of changed absorption. Of interest in this context is De Eds' suggestion that a possible mechanism for the effect of flavonoids on capillary resistance is one mediated *via* the pituitary–adrenal axis [3]. The plausibility of any 'enhanced absorption' theory is lessened by the finding that intramuscular injection of an orange peel flavonoid extract produced a similar elevation of tissue ascorbic acid as an orally-administered preparation [49]. In man, the results of a recent study have been interpreted as indicating a lack of evidence that flavonoids enhance absorption [63].

Stabilisation of ascorbic acid by flavonoids. Ascorbic acid, at physiological conditions of pH and temperature, undergoes a comparatively rapid breakdown [61]. Oxidative conversion to the first catabolite, dehydroascorbic acid, may occur by a number of enzymic and non-enzymic mechanisms and is catalysed by metallic ions, notably copper. Unless the dehydroascorbic acid is rapidly re-converted to ascorbic acid by chemical and enzymic systems present in the body

(see below), it undergoes further breakdown by irreversible pathways to give catabolites with no vitamin C activity [61]. Substances able to retard the tissue degradation of ascorbic acid would be expected to raise the tissue concentration of the vitamin. *In vitro* studies have indicated that flavonoids have a considerable capacity for retarding the breakdown of ascorbic acid to dehydroascorbic acid although the actual mechanism of this protection is not fully understood [7,64–66].

The traditional approach has been to accept that flavonoids retard the copper-catalysed oxidation of ascorbic acid by chelating with copper and possibly other trace elements. Harper, Morton and Rolfe have shown that the protective mechanism is possibly more complex than this; they found that flavonoids exerted a strong protective action under conditions where EDTA (a potent inhibitor of copper-catalysed ascorbic acid oxidation) was ineffective [67]. As an alternative and possibly complementary mechanism, they suggested that the protective capacity is derived from the ability of flavonoids to act as free radical acceptors; free radical formation is believed to be an important phase of ascorbic acid oxidation [67]. It should be noted, however, that the model system used for these studies was designed primarily to elucidate the mechanism of ascorbic acid protection by flavonoid in fruit juices at a low pH; it would be improper, without qualification, to extrapolate them to physiological conditions of pH, temperature and concentration.

Clemetson and Anderson have attempted to relate ascorbic acid-protective capacity to the structure of flavonoids [60]. They studied the effect of some 34 different flavonoids on the oxidation of ascorbic acid at physiological conditions and concluded that significant antioxidant activity was confined to compounds characterised by (a) the 3′,4′-catechol grouping ('couplet') of the B-ring and (b) the 3-hydroxy-4-carbonyl grouping ('couplet') of the γ-pyrone ring (*Figure 6.2*). In a more recent study (and in partial confirmation of this theory), quercetin and rutin were found to have a greater 'ascorbic acid-protection' capacity than the other flavonoids examined [49]. The apparent exception to the Clemetson and Anderson generalisation is hesperidin which does not confirm to the 'preferred pattern' and yet has *in vitro* protective capacity and *in vivo* increases the tissue ascorbic acid concentrations [44,46,49]; it has been claimed that many

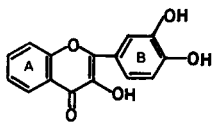

Figure 6.2. The postulated 'essential structure' for stabilisation of ascorbic acid by flavonoids [60]

commercial samples of hesperidin contain other flavonoids as impurities [60].

The extent to which enzymic systems are involved in the breakdown of ascorbic acid in tissues is not known although there is evidence for the existence of ascorbic acid-degrading enzymes such as dehydroascorbatase and 2,3-diketo-aldonate decarboxylase [68]. Flavonoids, possibly by chelating with essential trace elements are able to inhibit a wide range of enzymes [17]; inhibition of ascorbic acid-catabolizing enzymes is a theoretically possible mechanism whereby tissue ascorbic acid levels could be elevated. Of interest in the general context of inhibition of its breakdown are recent indications that ascorbic acid metabolites are mutagenic for microbial and mammalian cells [69]. An increased production of ascorbic acid metabolites in ageing systems could be a key factor in any intrinsic mutagenesis theory of ageing [70]. Flavonoids (and any other factors, such as thiol compounds, which depress the breakdown of ascorbic acid) would then possess anti-ageing properties; it would be a useful exercise to study experimentally the influence of dietary flavonoid supplements on the life-span of experimental animals.

Flavonoids and the reduction of dehydroascorbic acid to ascorbic acid. The half-life of dehydroabsorbic acid at physiological pH and temperature is about 4 min and unless it is reduced back to ascorbic acid it undergoes further and irreversible breakdown. Thiol compounds, and in particular glutathione, are potential hydrogen donors for this system (*Figure 6.3*). It has been suggested by French scientists that certain flavonoids ('vitamin P'), designated by them vitamin C_2 and which they regard as a second antiscorbutic factor, can potentiate this chemical reduction of dehydroascorbic to ascorbic acid. They have isolated such a vitamin C_2 factor from the liver phospholipoprotein fraction [71–74]. Wilson and Hughes (unpublished data) have confirmed that the reduction of dehydroascorbic acid by glutathione is enhanced by hesperidin, quercetin and

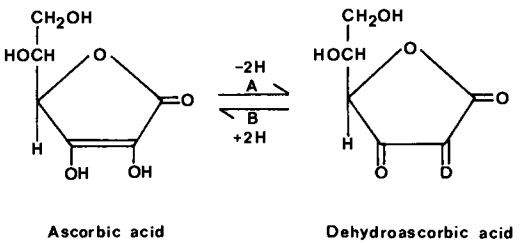

Figure 6.3. Ascorbic acid and dehydroascorbic acid. Oxidation A is catalysed by metallic ions, particularly copper, and is depressed by flavonoids; in reduction B, glutathione is the usual hydrogen donor and there is some evidence that the activity of the system is enhanced by flavonoids

rutin although the increase in reduction was somewhat less than that reported by the French workers under similar conditions.

Of interest in this context are reports of other factors able to potentiate the reduction of dehydroascorbate by glutathione. A well-characterised glutathione : dehydroascorbate oxidoreductase (EC 1.8.5.1) occurs in plants and in bacteria [75,76] and Grimble and Hughes have reported the existence of a functionally-similar factor in animal tissues [77,78], which, however, possessed a thermostability not normally associated with enzymes [78]. This animal tissues factor may be identical with, or a derivative of, the French workers' 'C_2 liver factor' and that it is a flavonoid or of flavonoid origin. This interpretation derives some support from the recent finding that quercetin produced a mild enhancement of the glutathione–dehydroascorbate system but it had no measurable effect on the activity of the liver factor described by Grimble and Hughes [78]. The possibility that flavonoids may stimulate the tissue reduction of dehydroascorbic acid is underlined in a recent publication by Zloch; guinea-pigs were given a standard amount of dehydroascorbic acid with and without flavonoids (rutin, epicatechin); the resultant tissue ascorbic acid was 30–100% greater in the case of the flavonoid-treated group [79].

Metabolic 'sparing' of ascorbic acid by flavonoids. The fourth possible mechanism for the flavonoid-induced increases in tissue ascorbic acid is that flavonoids, by taking over some of the biological functions of the acid, thereby reduce the 'metabolic demand' for the vitamin and permit its increased accumulation in the tissues. The inability of flavonoids to prolong the life of scorbutic guinea-pigs [43,46] implies that if 'substitution' occurs then it must be in an area other than that normally associated with the development of scurvy – namely, the defective hydroxylation of the collagen molecule precursor. A possible corollary of this is that ascorbic acid is a multifunctional vitamin, with functional substitution by flavonoids possible only in certain metabolic areas. Such 'partial substitution' is not unknown in nutrition; the partial replacement in animals of the vitamin E function of tocopherols by antioxidants is a case in point.

Related to, but distinct from, metabolic substitution or replacement is 'metabolic enhancement' where it is visualized that flavonoids could act synergistically with ascorbic acid. This would increase the biological potency of the acid but would not necessarily result in elevated concentrations of it in tissues. In areas where ascorbic acid has supposed metabolic involvement distinct from the simple prevention of scurvy, there is little evidence that flavonoids enhance or potentiate the ascorbic acid activity [80,81]. In a recent study of the influence of the acid on the incidence of the common cold in a group of 350 young persons, a daily dose of 80 mg 'synthetic' ascorbic acid significantly depressed by 18% the number of symptoms recorded; this too was the extent of the reduction in a parallel group on 80 mg of the acid as orange juice [59]. It would never-

theless be of value to determine whether flavonoids have any influence *in vitro* on the lysine–proline hydroxylation system – the only biochemical system where the vitamin has a clearly established role.

CONCLUSION

A complete assessment of the physiological significance of flavonoids is made difficult by a combination of factors such as the wide range of, often incompletely defined, flavonoid materials used by different workers, a comparative lack of knowledge of the factors influencing flavonoid absorption and metabolism, and little or no knowledge of the physiological significance of flavonoid-breakdown products on metabolites. Flavonoids, with their chelative and antioxidative capacity, may influence the metabolism of a wide range of substances of physiological significance in addition to ascorbic acid which, presumably for historical reasons, is the nutrient most frequently regarded as having a close relationship with flavonoids. Lipids and adrenaline are examples of compounds known to be protected against oxidation by flavonoids, and the currect nutritional tendency to attribute flavonoid-induced changes in physiological patterns primarily to changes in ascorbic acid status alone is scientifically unjustified. For the same reason, any nutritional influence exerted by flavonoids could be related to, and modified by, the nature of the diet, particularly its lipid content.

Despite these methodological difficulties – exacerbated in man by the additional problem of obtaining a flavonoid-free diet – there are grounds for believing that flavonoids influence capillary resistance. Available evidence suggests that they are of greater significance in 'correcting' deviant patterns and in restoring a physiological *status quo* than in maintaining the normal integrity of the capillaries, i.e. their mode of action is pharmacological rather than nutritional.

Nevertheless, there are indications that flavonoids may, in certain clearly defined areas, be of nutritional significance – in the sense that they may influence the fate of nutrients in the body. Of particular interest is their effect on the growth and metabolism of hypovitaminotic-C guinea-pigs. This is a puzzling relationship for which no generally accepted explanation has been proferred. Nor is it clear whether this represents a direct flavonoid–ascorbic acid interaction or whether hypovitaminosis C merely highlights, in a currently indefinable way, a separate relationship between e.g. protein and flavonoids. Perhaps Kuhnau's description of flavonoids as 'semi-essential' nutrients has scientific implications that outweigh its semantic unacceptability!

Future studies should seek to examine the wider and long-term implications of flavonoid–metal chelation – not only in the context of ascorbic acid stability but in terms of cellular availability of essential trace metals. The influence of

dietary flavonoid on the life span and reproductive capacity of small animals could be a useful contribution in this respect [82]. In more overtly nutritional terms, the relationship between flavonoids and ascorbic acid merits further examination. The possibility that flavonoids may inhibit the breakdown of tissue ascorbic acid could be of some physiological significance, particularly in elderly subjects. There is a well-established negative correlation between age and tissue levels of the vitamin in both man and guinea-pigs to which an increased degree of tissue breakdown of the acid could be a contributing factor. Flavonoids by inhibiting ascorbic acid breakdown and thereby reducing the mutagenic potential of ageing tissues could, theoretically, be of some significance in both the ageing process and the incidence of disease.

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