PROGRESS IN MEDICINAL CHEMISTRY

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PREFACE

We are pleased to present six reviews in the fifth volume of this series. The opening chapter on polypeptide antibiotics is complementary to a review of other medicinally important polypeptides which appeared in Volume 4; these two reviews together include references to over 1250 papers, and give an indication of the tremendous effort that has been concentrated in this field in recent years. Another very active subject—non-steroidal anti-inflammatory drugs—is reviewed in Chapter 2; although research in this field has recently yielded some useful drugs, several aspects of the mode of action and structure–activity relationships remain unsolved.

The next two chapters emphasize the biochemical aspects of heparin and heparinoids, and of histidine decarboxylases. Chemical elucidation of the structure of heparin derived from different species is now in sight, and it is hoped that this advance will hasten progress into the true physiological function of this mucopolysaccharide; further analyses of the characteristics of the two histidine decarboxylases may also soon indicate their physiological importance, especially in the control of the microcirculation. The chapter on psychotropic drugs and neurohumoral substances follows, and attempts to cover the advances in our knowledge of chemical transmission within the central nervous system so that psychotropic drugs can be used in the future against a more scientific background.

Finally, the chemical, bacteriological and clinical aspects of the nitrofurans are reviewed in Chapter 6 with special emphasis on the significant Japanese contribution to this field. Research continues unabated in an effort to discover a drug which retains the very desirable antibacterial properties of the existing nitrofurans but which lacks some of the disadvantages that limit their clinical application.

It is hoped that readers will appreciate the effort that our authors have made to cover as thoroughly as possible the ever-expanding literature of their respective subjects. We thank reviewers for their encouragement, the staff of Butterworths for their help, and authors, societies and publishers for permission to reproduce material. We apologize to Dr. P. S. J. Spencer for inadvertently omitting from the contents list of Volume 4 his qualifications (B.Pharm., Ph.D.) and his address (at the time: Allen & Hanburys Ltd., Ware, Herts).

G. P. Ellis
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POLYPEPTIDE ANTIBIOTICS OF MEDICINAL INTEREST

R. O. STUDER

INTRODUCTION

IN the 26 years since the discovery of tyrothricin—the first peptide antibiotic isolated—more than a hundred antibiotic substances containing one or more amino acids or moieties derived from amino acids have been described. However, despite considerable efforts, the structures of relatively few peptide antibiotics have been determined and ultimately proved by synthesis. There are several reasons for this.

First, many of these antibiotics, after their discovery, characterization and evaluation, were not of sufficient practical importance and work on them was discontinued. Second, they have relatively complicated structures. Unlike the peptide hormones, which are composed of a limited number of natural amino acids found in proteins, many peptide antibiotics contain a large variety of components other than amino acids. Besides the frequently occurring amino acids not isolated from proteins (*Table 1.1*), many other components, such as hydroxy acids, amino sugars, fatty acids, pyrimidine derivatives and so on, are present (*Table 1.2*). They also often contain unusual peptide linkages, unknown in proteins. Furthermore, most of the peptide antibiotics are of cyclic nature and for this reason are resistant to enzymatic attack, which makes their structural elucidation in many instances more complicated.

A third reason for this lack of knowledge may be the present limited therapeutic importance of the peptide antibiotics, mainly as a result of their narrow antimicrobial spectrum and their relatively high toxicity. However, the recent increase in serious infections caused by gram-negative bacteria and their growing resistance to the broad spectrum antibiotics has turned interest to some of the peptide antibiotics, which are the most active inhibitors of these organisms.

CLASSIFICATION OF PEPTIDES

Peptides can be classified according to several principles. With regard to chemical characteristics, Bricas and Fromageot[1] have divided the peptides into two groups: the *homoeomeric peptides*, which are built up only of amino acids, and the *heteromeric peptides*, which contain other components beside amino acids. Most peptide hormones belong to the homoeomeric peptides, while the majority of the peptide antibiotics belong to the heteromeric peptides. This classification has been extended[2,3] to the cyclopeptides by dividing them into the *homodet* and *heterodet cyclic peptides*. The former are peptides with rings formed only from amino acids in peptide linkages, whereas the rings of the latter contain other linkages also. A group of peptide-related

Table 1.1. Amino acids found in peptide antibiotics

Amino acids found in antibiotics and proteins	*Related amino acids found only in antibiotics*
L-alanine	*N*-methyl-L-alanine, β-alanine, L-β-(2-thiazolyl)-β-alanine
L-arginine	
L-aspartic acid	D-aspartic acid, L-β-methylaspartic acid
L-cysteine	D-cysteine, *N,N'*-dimethyl-L-cystine
L-glutamic acid	D-glutamic acid
Glycine	Sarcosine, L-α-phenylsarcosine, L-phenylglycine, α-(2-iminohexahydro-4-pyrimidyl)-glycine
L-histidine	
L-hydroxyproline	Allo-D-hydroxyproline, *cis*-3-hydroxy-L-proline, *trans*-3-hydroxy-L-proline
L-isoleucine	D-isoleucine, allo-D-isoleucine, *N*-methyl-L-isoleucine
L-leucine	*N*-methyl-L-leucine, β-*N*-dimethyl-L-leucine, L-β-hydroxyleucine, D-leucine
L-lysine	L-β-lysine
L-methionine	
L-phenylalanine	D-phenylalanine, *N*-methyl-L-phenylalanine, *N*-methyl-L-*p*-dimethylaminophenylalanine, 3-nitro-4-hydroxy-L-phenylalanine
L-proline	γ-methyl-L-proline, β-methyl-L-proline
L-serine	D-serine
L-threonine	Allo-L-threonine
L-tryptophan	D-tryptophan, β-methyl-L-tryptophan
L-tyrosine	
L-valine	D-valine, *N*-methyl-L-valine, L-norvaline, L-γ-formylmethylnorvaline
	L-ornithine, D-ornithine, *N*-hydroxy-L-ornithine
	L-lanthionine, β-methyl-L-lanthionine
	L-α-aminobutyric acid, D-α-aminobutyric acid, L-α,γ-diaminobutyric acid
	L-α-amino-β-phenylbutyric acid, α, β-diaminobutyric acid
	L-α, β-diaminopropionic acid, β-aminopropionic acid
	δ-aminovaleric acid, 2-aminohexenoic acid, 6-diazo-5-oxo-aminohexanoic acid
	D-α-aminoadipic acid
	1-methyl-4-aminopyrrole-2-carboxylic acid, 1-methyl-5-aminopyrrole-2-carboxylic acid
	D-penicillamine, streptolidine, 3-aminosalicylic acid
	D-α-pipecolic acid, 4-oxopipecolic acid, 2-(1-amino-2-methylpropyl) thiazole-4-carboxylic acid
	Thiostreptoic acid, thiostreptine

Table 1.2. Components other than amino acids found in polypeptide antibiotics

Amines

Ethanolamine
Cadaverine
1-amino-5-hydroxylaminopentane

Sugars

D-gulosamine
Glucose, xylose
Unidentified sugars

Other components

3-methyluracil, β-picoline, Fe

Acids

Formic acid
Propionic acid
D-α-hydroxyisovaleric acid
α-n-hexyl-β,γ-dihydroxypentanoic acid
6-diazo-5-oxohexanoic acid
6-methylheptanoic acid
(+)-6-methyloctanoic acid
3-hydroxydecanoic acid
β-hydroxytridecanoic acid
(+)-12-methyltetradecanoic acid
13-methyltetradecanoic acid
Lactic acid
Guanidino-acetic acid
Succinic acid
3-hydroxypicolinic acid
2-propionylthiazole-4-carboxylic acid
Quinoxaline-2-carboxylic acid
Actinocinin
Viomycidine

antibiotics, which are built up of hydroxy and amino acid residues joined by amide and ester bonds, are known as *depsipeptides*[4–8]. The antibiotics with known structures are listed and classified in this way in *Table 1.3.*

For this review another classification is used. As the majority of peptide antibiotics are of unknown structure and cannot therefore be classified chemically, they are grouped according to the organism which produces them. At the beginning of every section, the peptide antibiotics are listed in a table which gives the known characteristics and the literature. Only the clinically useful members are described in detail. Furthermore, only those peptide antibiotics which consist mainly of amino acids in peptide linkages are fully considered. The important actinomycins[9], penicillins[10–13] and cephalosporins[14], which have been reviewed several times in recent years, are not included here. Amino acids and amino acid residue sequences are denoted in accordance with the suggestions of the committee on nomenclature which reported at the Fifth European Peptide Symposium[15]. When necessary the direction of the —CO·NH— bond is indicated by an arrow (→).

Every effort has been made to cover the work published up to the end of 1964.

TECHNIQUES OF PEPTIDE CHEMISTRY

Methods of Structural Elucidation

The methods employed to deduce the structures of peptides have been reviewed by several authors[16–19] and are not commented on here. These methods, when applied to peptides of animal origin with their limited number of amino acids, can be used with a minimum amount of peptide and the released amino acids or their reaction products can usually be identified unequivocally by chromatographic procedures. In the case of peptide antibiotics with their many uncommon constituents, the characterization of these components is an additional task. Even the amino acids themselves need a careful investigation. They may be derivatives of common amino acids, they may be secondary degradation products from unusual components, or they may have D-configuration. Therefore the components have usually to be isolated in amounts which allow a careful chemical and physicochemical characterization, and often direct comparison with synthetic compounds is necessary.

Another difficulty often encountered in the field of peptide antibiotics is the proof of homogeneity. Most of these antibiotics are produced as a group of closely related compounds, which are difficult to separate. Here, extensive countercurrent distribution[20] and the method of multiple dialysis[21] are very valuable.

The determination of the molecular weight has also often led to difficulties. Freezing-point depression, diffusion and sedimentation in the ultracentrifuge have sometimes led to discrepancies owing to association or solvents of crystallization. The partial substitution method of Battersby and Craig[22], the thermoelectric method of Simon and Tomlinson[23], mass-spectrometric measurements and X-ray analysis have been used recently to overcome such difficulties.

Final evidence for the accuracy of the proposed structure is usually

Table 1.3.

Peptide antibiotics

- *Homoeomeric peptides*
  - *Linear peptides*
    - None
  - *Cyclopeptides*
    - *Homodet cyclic*
      - Bacitracin A
      - Gramicidins
      - Ilamycins
      - Mycobacillin
      - Tyrocidine A, B, C, D
    - *Heterodet cyclic*
      - Telomycin
- *Heteromeric peptides*
  - *Linear peptides*
    - Gramicidin A, D
  - *Cyclopeptides*
    - *Homodet cyclic*
      - Albomycin
      - Circulin A
      - Colistin A, B
      - Polymyxin B_1, B_2
    - *Heterodet cyclic*
      - Actinomycins
      - Echinomycin
      - Enniatin A, B, C
      - Esperin
      - Etamycin
      - Serratamolide
      - Staphylomycin S
      - Vernamycins
      - Valinomycin

obtained by synthesis and careful chemical, physicochemical and biological comparison with the natural product. However, synthesis in the field of peptide antibiotics is a rather laborious task and has been achieved only in a few cases. Other methods have therefore been used to provide information about the correctness of a proposed structure. The nuclear magnetic resonance spectra in deuterio-acetic acid[24,25], mass-spectrometric studies[26,27], and optical rotatory dispersion of metal complexes[28] have proved helpful.

Methods of Synthesis

The methods of peptide synthesis have been reviewed by several authors within the last few years[29–36]. Special methods of peptide synthesis have been discussed annually since 1958 at the European Peptide Symposium and are published in the proceedings of these meetings[15,37–42]. It is therefore not intended to give a survey here.

The methods for the synthesis of cyclopeptides have been reviewed by Schwyzer[43] and by Rothe[44]. For the synthesis of a homodet cyclic peptide, a corresponding suitably protected linear peptide is usually prepared first. Three procedures are then available for the cyclization of these key peptides. In a first method both the *N*-terminal and the *C*-terminal protecting groups are removed and the resulting free peptide is treated with carbodi-imide[45] in high dilution. In a second procedure an active peptide is prepared by transforming the carboxyl group into an activated ester[43,44] or an azide[46], which after removal of the *N*-terminal protecting group is submitted to cyclization in high dilution. Peptide azides may also be prepared directly from the free peptide hydrazides[47,48]. A third procedure, the activation of the amino group of a free peptide[49], has rarely been used.

For the synthesis of the heterodet cyclic depsipeptides, the acid chloride which had been prepared after the removal of the *N*-protecting groups[50,51] was most used. Recently, a mixture of acetylchloride and acetylimidazole has been used for the cyclization of actinomycin C_1 acid to actinomycin C_1[51a]. Heterocyclic peptides containing disulphide rings have been cyclized by oxidation of the corresponding disulphydryl compounds[52].

Doubling reactions[53,54], in which two molecules of the activated peptide condense prior to cyclization, have been observed on several occasions, and have been used for the synthesis of gramicidin S[53]. Because of these side-reactions, the careful characterization of the end-products, especially the determination of the molecular weights, is of paramount importance.

POLYPEPTIDE ANTIBIOTICS PRODUCED BY *B. Brevis*

General Considerations

Brevin, Brevolin and Edein, three peptide antibiotics with structures yet unknown, are not in clinical use (*Table 1.4*). Tyrothricin consists of a whole group of chemically and biologically closely related individuals, a feature known for the polymyxins, the bacitracins and other peptide-type antibiotics. The tyrothricin group is interesting not only because of its clinical application, but also from a historical viewpoint. Tyrothricin was the second antibiotic found, 10 years after the discovery of penicillin, in a direct search for antibiotic substances. As the first antibiotic of strictly peptide nature, it

is closely linked to the development of the modern techniques of peptide chemistry. Paper-chromatography, ionophoresis, countercurrent distribution, partial hydrolysis, end-group analysis by Sanger, molecular weight determination and even synthesis of cyclopeptides were tried on these compounds and proved their usefulness.

From the biogenetic viewpoint, the tyrothricins are interesting because they contain, unlike the other antibiotic families, two different types of peptides: on the one hand the cyclic tyrocidines, and on the other the linear gramicidins A, B, C and D, which are also protected on the amino end by the unusual formyl group and by an ethanolamine residue on the carboxyl end. The latter are the only non-cyclic antibiotics known to the present day (*Table 1.4*).

Gramicidin S is closely related to the tyrocidines since it contains one of the pentapeptide sequences of tyrocidine repeated twice in its cyclic molecule.

Tyrothricin

Discovery and nomenclature

In 1939 Dubos reported the isolation of the antibiotic tyrothricin from culture filtrates of strains of *Bacillus brevis*[58,59,90]. He and his co-workers[91,92] soon succeeded in separating tyrothricin with organic solvents into two crystalline compounds, the neutral gramicidin and the basic tyrocidine (formerly called graminic acid and gramidinic acid). Tyrothricin is thus a term for the partially purified antibiotic obtained from the culture fluids of *Bacillus brevis*. It contains on the average a mixture of 20 per cent gramicidin and 80 per cent tyrocidine. Besides these, at least three further polypeptides have been found by countercurrent distribution of tyrothricin.

Chemistry

The tyrocidines—The peptide nature of tyrocidine soon became apparent and several components were identified[60,93,94]. This early period has been reviewed by Hotchkiss[60] and by Synge[61]. A big step toward the elucidation of the structure was made in 1949, when American workers[62,64] were able to demonstrate that the crystalline tyrocidine hydrochloride was not a homogenous compound. It was fractionated by countercurrent distribution into three major crystalline components, called tyrocidine A, B and C[62], thus confirming an earlier indication of heterogeneity[95]. On hydrolysis, all three gave distinct spots on paper-chromatograms corresponding to phenylalanine, leucine, tyrosine, valine, proline, ornithine, glutamic acid and aspartic acid. In addition, B and C contained tryptophan.

The molecular weight of tyrocidine A was found to be about 1,270[96]. Total hydrolysis and quantitative amino acid analysis revealed the exact composition[62]. Partial hydrolysis, fractionation of the resulting peptide mixture by countercurrent distribution, ion-exchange chromatography and paper-chromatography, followed by sequential analysis, led Paladini and Craig to the structure of tyrocidine A[63] (*see Table 1.6*).

The structure of the crystalline tyrocidine B was elucidated shortly afterwards in a series of analogous experiments by King and Craig[65]. The only

Table 1.4. Polypeptide antibiotics produced by *B. Brevis*

Name	*Antibiotic spectrum*	*Mol. wt.*	*Amino acids**		*Other components*	*References*
			D	L		
Brevin	Gram-pos., bacteria		Asp, Gly, Ser, Tyr		Unknown base	55
Brevolin	Gram-pos., gram-neg.		Arg, Leu, Tyr, Val, several unknown			56
Edein	Gram-pos., gram-neg.		Arg, Glu, Gly, Tyr, two unknown			57
Tyrothricins	Gram-pos., gram-neg.					58–61
Tyrocidine A	Gram-pos., gram-neg.	~1,270	Phe	$Asp(NH_2)$, $Glu(NH_2)$, Leu, Orn, Phe, Pro, Val, Tyr		62–64
Tyrocidine B	Gram-pos., gram-neg.	~1,310	Phe	$Asp(NH_2)$, $Glu(NH_2)$, Leu, Orn, Pro, Try, Tyr, Val		62, 64, 65
Tyrocidine C	Gram-pos., gram-neg.	~1,350	Phe, Try	$Asp(NH_2)$, $Glu(NH_2)$, Leu, Orn, Pro, Tyr, Val		62, 64, 66
Tyrocidine D	Gram-pos., gram-neg.	~1,390	Try	$Asp(NH_2)$, $Glu(NH_2)$, Leu, Orn, Pro, Tyr, Val		66
Gramicidin A	Gram-pos. bacteria	1,882	Leu, Val	Ala, Gly, Try, Val	Ethanolamine, formic acid	61, 64, 67–73
Gramicidin B	Gram-pos. bacteria	~2,000	Ala, Gly, Leu, Phe, Try, Val		Ethanolamine	61, 64, 67, 68
Gramicidin C	Gram-pos. bacteria	~2,000	Ala, Gly, Leu, Try, Tyr, Val		Ethanolamine	61, 64, 67, 68
Gramicidin D	Gram-pos. bacteria	1,897	Leu, Val	Ala, Gly, Ile, Try, Val	Ethanolamine, formic acid	70, 72, 73
Gramicidin S (J_1, J_2)	Gram-pos. bacteria	1,140	Phe	Leu, Orn, Pro, Val		74–89

* Whenever the configuration of the constituent amino acids has been determined, the D- and L-forms are separated by a vertical line.

remaining uncertainty concerned the asparagine- and glutamine-residues because there was no direct evidence that iso-asparagine and isoglutamine were not involved[65].

Only recently have structures been proposed[66] for tyrocidine C and for the newly isolated tyrocidine D.

The gramicidins—Soon after the isolation of the crystalline gramicidin, Hotchkiss[93] presented evidence of its peptide nature and identified several components after total hydrolysis. He found neither a free amino nor carboxyl group. Gordon, Martin, and Synge determined the amino acid composition[97] and identified 2-aminoethanol[98] as an additional constituent. These early experiments for the characterization of gramicidin have been reviewed by Synge[61,67]. Recrystallization[99], diffusion[100] and adsorption[95] studies did not reveal any heterogeneity in gramicidin. Craig, Gregory and Barry[64,68] were able to demonstrate, by the newly developed method of countercurrent distribution, that gramicidin is a mixture of at least three closely related substances. They thus introduced a new criterion of purity, which was also of prime importance for the characterization of this group of substances. The three components, in order of increasing partition coefficient, were called gramicidin B, A and C. Besides these, there was indication of at least one more component. The three gramicidins, isolated in crystalline form, were found to differ in their amino acid content, both qualitatively and quantitatively[64,68].

More than 10 years later, indication was obtained[69] that gramicidin A in itself is heterogenous. When gramicidin A was submitted to countercurrent distribution over 2,000 transfers[70], a small peak, called gramicidin D, separated from the main peak, still designated as gramicidin A. Even after this extensive purification the main component gramicidin A may still be contaminated[70]. By total hydrolysis and quantitative analysis the composition of the different gramicidins was determined (*Table 1.5*), showing clearly their close relationship[70].

Table 1.5. Composition of the different gramicidins[70]

Name	*Partition coefficient*	*Amino acids*	*Other Components*
Gramicidin A	0·70	$Gly_1Ala_2Val_4Leu_4Try_6$	$Ethanolamine_1$ Formic acid
Gramicidin B	0·32	$Gly_1Ala_2Val_4Leu_4Phe_1Try_{3-4}$	$Ethanolamine_1$
Gramicidin C	1·41	$Gly_1Ala_2Val_4Leu_4Tyr_1Try_6$	$Ethanolamine_1$
Gramicidin D	0·59	$Gly_1Ala_2Val_3Ile_1Leu_4Try_6$	$Ethanolamine_1$ Formic acid

Recently it has been found that the former gramicidin A (mixture of A and D) contains a formyl group, which blocks the *N*-terminal amino group[71,72]. This protecting formyl group can be removed by treatment with 1·5N anhydrous hydrogen chloride in absolute methanol. By subsequent *N*-terminal amino acid analysis according to the method of Sanger, they found valine and isoleucine, thus confirming the heterogeneity of the former crystalline gramicidin A. These results, obtained by Edman degradation, in combination with earlier findings[73,101,102] led to the structure (*Table 1.6*) for gramicidin A (valine-gramicidin A) and for gramicidin D (isoleucine-gramicidine A)[72]. These two formulae best express all published observations up to now and also resolve the doubts about the molecular weight of gramicidin A[67]. They establish a molecular weight of 1,882 for gramicidin A (valine-gramicidin A), a value now confirmed by ultracentrifuge studies[72].

Gramicidin A is not attacked by enzymes such as nagarse, pronase, chymotrypsin and pepsin[71].

Gramicidin S—Another member of the tyrothricin group is gramicidin S ('Soviet gramicidin'), isolated in 1944 in crystalline form from an organism similar to *B. brevis*[74,75]. It was immediately apparent that this antibiotic differed from the gramicidin of Dubos in its biological and chemical aspects[74]. The antibiotic was characterized as a polypeptide by the Russian authors, who also identified proline, ornithine and leucine after total hydrolysis[75]. Synge[76] suggested in 1945 that gramicidin S is probably a cyclodecapeptide having a stoichiometric minimum unit formed from one residue each of L-ornithine, L-proline, L-valine, L-leucine and D-phenylalanine. These data made it clear that gramicidin S is closely related to tyrocidine. The presence of two free amino groups contributed by the δ-amino group of the ornithine residue and the absence of any free carboxyl group supported the proposed cyclic nature[77]. Consden and his co-workers[78,79] fractionated the products of partial hydrolysis by the use of two-dimensional paper-chromatograms and ionophoresis. They proposed the sequence L-valyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-prolyl, which occurs once or repeatedly in a cyclopeptide. Diffusion[100] and cryoscopic[75] measurements, countercurrent distribution of the 2,4-dinitrophenyl derivatives[80] and x-ray examination of a series of derivatives of gramicidin S[81–84] favoured a molecular weight consistent with a decapeptide consisting of two pentapeptides of the established sequence (*Table 1.6*).

In 1959, Japanese workers[85,86] reported the isolation and structure of a new gramicidin, designated gramicidin J_1, and in the same year Noda[87] isolated and determined the structure of gramicidin J_2. The total synthesis of gramicidin J_2 was published[87], but recently these findings were revised and the identity of gramicidin J_1 and gramicidin J_2 with gramicidin S was firmly established[88].

Synthesis

The only member of the tyrothricin group synthesized to date is gramicidin S. Harris and Work[103] had prepared several pentapeptide derivatives. Later, syntheses of protected pentapeptides[104,105] and decapeptides[105] were published, but the final synthesis, marking the first synthesis of a naturally occurring cyclic peptide, was accomplished by Schwyzer and Sieber[89].

Table 1.6. The structures of the members of the tyrothricin group

	X	Y	Z
Tyrocidine A	D-Phe	L-Phe	D-Phe
Tyrocidine B	D-Phe	L-Try	D-Phe
Tyrocidine C	D-Try	L-Try	D-Phe
Tyrocidine D	D-Try	L-Try	D-Try

Gramicidin S

HCO-X-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Try-D-Leu-L-Try-D-Leu-L-Try-D-Leu-L-Try-NHCH$_2$CH$_2$OH

Gramicidin A (valine-gramicidin A) X = L-Val
Gramicidin D (isoleucine-gramicidin A) X = L-Ile

First the repeating pentapeptide unit (*Figure 1.1*) was built up. Removal of either the *N*-terminal trityl group or the *C*-terminal methyl group and connection of the two resulting pentapeptides by means of carbodi-imide yielded the protected linear decapeptide. This was converted to the corresponding *p*-nitrophenyl ester by saponification of the methyl ester and reaction with di-(*p*-nitrophenyl)sulphite. After removal of the *N*-terminal trityl group the cyclization was achieved by reaction in hot pyridine. Finally the tosyl groups which protected the δ-amino groups of ornithine throughout the synthesis were removed and the product was purified by countercurrent distribution. It proved to be identical with natural gramicidin S in all respects.

In a later attempt to prepare the cyclic pentapeptide by cyclization of the *p*-nitrophenyl ester of the pentapeptide sequence, Schwyzer and Sieber[106] isolated the cyclized decapeptide. Two pentapeptide derivatives had condensed and cyclized, a doubling reaction later observed with other cyclic peptides.

Swiss workers have reported the syntheses of a protected pentapeptide sequence[107] and of a protected decapeptide sequence[108] of tyrocidine A. The final cyclization has yet to be achieved.

Antimicrobial activity

Tyrothricin[60,109] is bacteriostatic in concentrations of 0·01 μg/ml. and less for gram-positive cocci such as pneumococci, streptococci and staphylococci. At concentrations greater than 1 μg/ml. it is bactericidal and some species of organisms, e.g. pneumococcus and staphylococcus are lysed. Gram-negative bacteria are much less sensitive. Many penicillin-resistant strains of cocci are sensitive to tyrothricin (*Table 1.7*).

Gramicidin[60,109] is bacteriostatic and to some degree bactericidal in concentrations of 0·01 to 1 μg/ml. for gram-positive cocci. Even at very high doses there is no lysis of any organism. It has no activity towards gram-negative organisms.

Tyrocidine[60,109] has about one-tenth to one-fifteenth of the activity of gramicidin against gram-positive cocci, but it is somewhat more active against gram-negative cocci and bacilli. It is responsible for the activity which tyrothricin shows against the gram-negative intestinal bacilli, whereas gramicidin is responsible for the high activity of tyrothricin against the gram-positive organisms. At bactericidal concentrations tyrocidine produces lysis in some species of organisms. Tyrocidine as well as gramicidin is haemolytic[110]. Tyrocidine is inactivated by peptone, blood serum, and body fluids and therefore gramicidin, which is not affected in its activity, is for all practical purposes the active principle when these are present.

Gramicidin S is less selective than tyrothricin in its antibacterial action. It is not only highly active towards gram-positive organisms, but also prevents growth and kills many varieties of gram-negative bacteria at 25 to 50 μg/ml.; among these are *Proteus vulgaris* and *Escherichia coli*[75].

For the determination of potency of tyrothricin the turbidimetric assay method, with *Streptococcus faecalis* (group D) strain M 19 (ATCC 10 541) as test organism, is used. The reference standard is a mixture of 20 per cent crystalline gramicidin and 80 per cent crystalline tyrocidine hydrochloride[111].

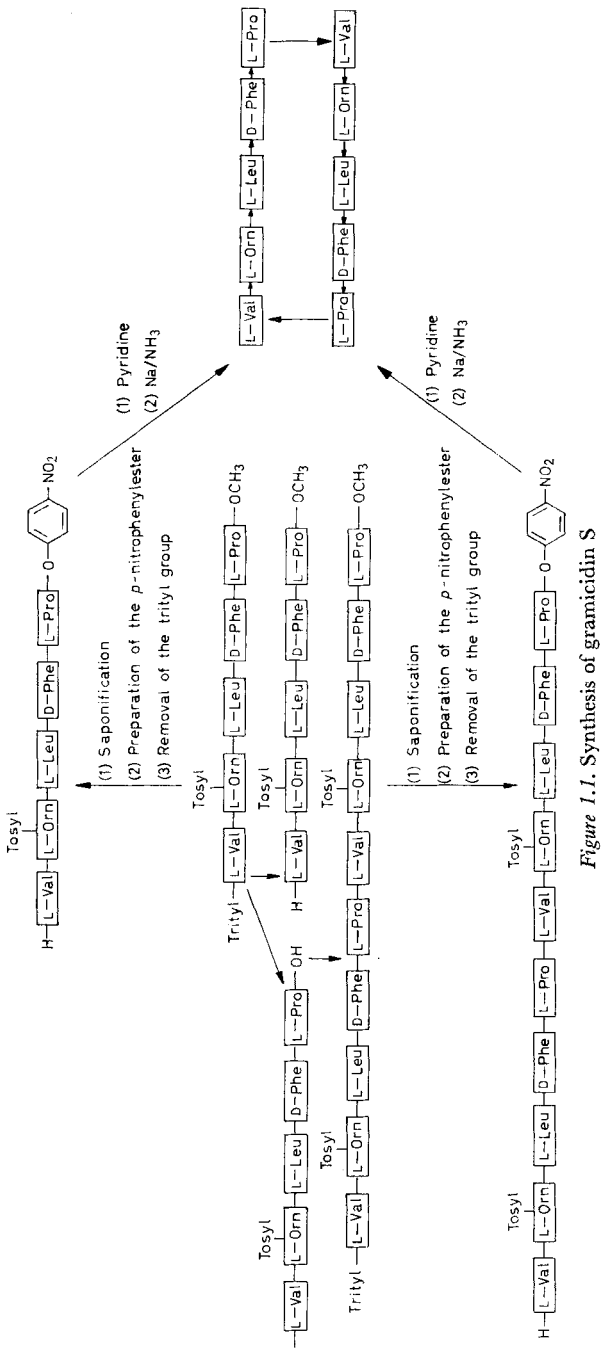


Figure 1.1. Synthesis of gramicidin S

Table 1.7. Antimicrobial activity of the peptide antibiotics against some representative organisms (in μg/ml)*

Organism	*Amphomycin*	*Bacitracin*	*Tyrothricin*	*Gramicidin*	*Tyrocidine*	*Gramicidin S*	*Colistin*	*Polymyxin B*	*Viomycin*
Gram-positive									
Clostridium tetani		0·1–0·2	0·2	>20	2–10	7	>100	>100	
Corynebacterium diphteriae	0·25	0·1–0·3	10–50	>50			0·4	0·14	10–25
Diplococcus pneumoniae	2·5	0·05–2	3	0·1–20	2·5	10	>50	>50	50
Mycobacterium tuberculosis			0·03	20–50					2·5–12
Staphylococcus aureus	2–6	1–5	0·5–200	>100	8		100	100	
Streptoccocus pyogenes	2·5	0·01–2	2	1–50	3–10	~10	33	11	>80
Gram-negative									
Aerobacter aerogenes	>50	>100		>300	>300		0·3–1·2	3	25
Brucella							0·1–1	0·1–1	>50
Escherichia coli	>100	>100	>100	>300	>300	25	0·01	0·02	10–100
Haemophilus		13					0·25–10	0·25–10	12–50
Klebsiella pneumoniae	>100	>100					0·02	0·02	10–20
Neisseria meningitis		0·14	0·5–10	>100	40		3–33	>100	2·5–25
Proteus vulgaris	>100	>100				100	>100	>100	>100
Pseudomonas aeruginosa	>100	>100					0·1–4	0·02–4	>100
Salmonella typhi	>100	>100	>100	>300	800	50	0·01	0·02	10–25
Shigella	>100	>50		>300		50	0·01	0·02	100

* Owing to differences in assay techniques the values reported in the literature differ from each other. Average values from the literature mentioned in the text have been compiled mainly as an indication of strain specificity.

Structure–activity relationship

For the gramicidins, whose structures have been elucidated only recently, practically nothing can be said with regard to structure–activity relationship. Removal of the *N*-protecting formyl group reduces the antimicrobial activity to 60 per cent of that of gramicidin[71]. The antimicrobial activities reported for the different tyrocidines seem to indicate that phenylalanine may be replaced by tryptophan without greatly affecting the activity[112].

A great variety of analogues and derivatives of gramicidin S (gramicidin J_1 and J_2)[113–117] has been reported, but in most instances with little information concerning their activity and, especially, their toxicity.

Open-chain penta-[103] and decapeptides[105,118–120] and some of their analogues[118–120] possess some antimicrobial activity. Their mechanism of action, however, is different from that of gramicidin S[120]. Activity has also been reported for co-polymers of the amino acids occurring in gramicidin S and related amino acids[121]. The exchange of ornithine for lysine[107] or arginine[122] in gramicidin S leaves the activity practically unaltered. Preparation of alkyl[123] and peptide[124] derivatives under retention of the basic properties yielded substances with activities comparable to gramicidin S, whereas acylation[123], carboxymethylation[125] or replacement of the N^{γ}-amino groups by hydroxyl groups[123] resulted in great loss of activity. The exchange of proline for glycine[126] enhances the activity, while replacement of both proline and valine by glycine[127] results in drastically reduced activity. A cyclohexapeptide, cyclo-(L-Val-L-Orn-L-Leu-D-Phe-L-Pro-Gly-)[126] has no activity at all. Introduction of D-aminophenylalanine[128] instead of D-phenylalanine leads to a nearly inactive substance.

One might tentatively conclude from these results that the whole character of the molecule is responsible for the action and that any alteration of the ratio between hydrophobic and hydrophilic groups affects the antimicrobial activity. Several workers[43,129,130] have proposed molecular models for gramicidin S. Although they differ in details, they clearly show that the hydrophobic side-chains of valine, leucine and phenylalanine are aggregated on one side of the molecule, whereas the hydrophilic N^{γ}-amino groups of ornithine are situated on the other side. Such a conformation may explain the mechanism of action of gramicidin S as a surface active agent.

Clinical use

Tyrothricin as well as gramicidin, the mixture of the gramicidins A, B, C and D isolated from the tyrothricin complex, have been used for many years clinically in similar types of infections. Owing to their high toxicity (*Table 1.8*) their application is limited, and systemic (i.e. parenteral) use is never recommended. Like the other polypeptide antibiotics they are, however, non-toxic when administered orally or topically. Both are in use either alone or in combination with other antibiotics[131–135] in a great variety of ointments and solutions for the topical treatment of superficial ulcers, abscesses of the skin, mastoiditis, tonsillitis, dermatoses, wounds and burns, and infections of the eye and conjunctiva, where the predominating organisms are gram-positive. They usually exert no effect if they are not in direct contact with the organisms. As long as there is no direct contact with the blood stream,

Table 1.8. Comparative LD_{50} values of the peptide antibiotics for mice (mg/kg)

	Intravenous	*Intraperitoneal*	*Subcutaneous*	*Oral*	*References*
Amphomycin	178			>500	137
Bacitracin	210	300–700	1,200–3,300	>3,000	138, 139
Colistin	6	20	50	700	140, 141
Gramicidin (LD_{100})	2·5	40		>1,000	60
Gramicidin S (rats)		17			74
Polymyxin B	6	20	50	700	140, 141
Tyrocidine	15	40		>1,000	60
Tyrothricin	3·7	20		>1,000	60
Viomycin	240		1,380	>7,500	142

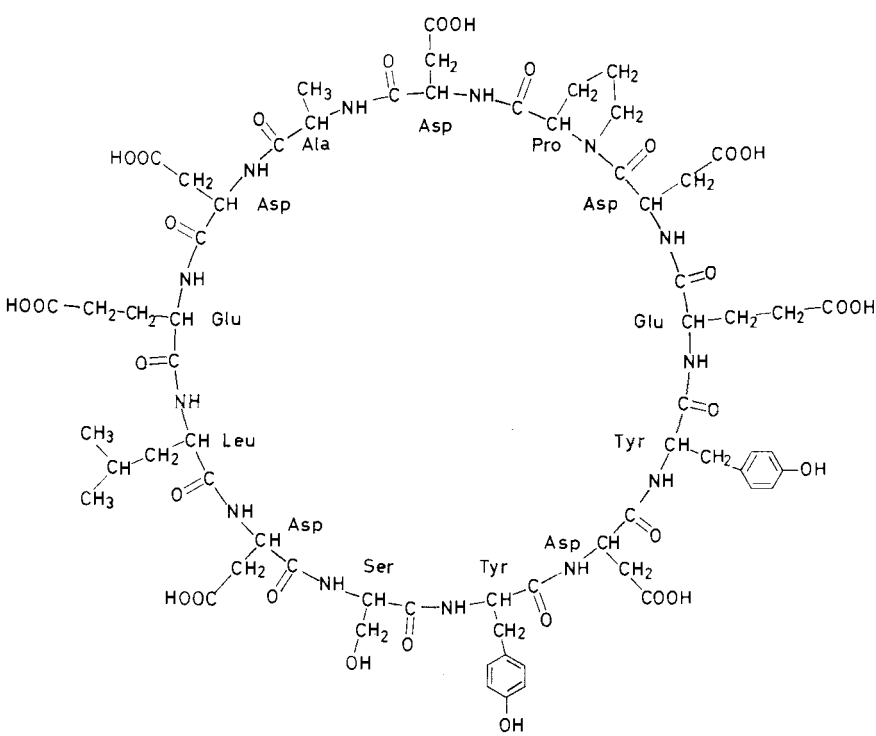


Figure 1.2. Proposed structure of mycobacillin

Table 1.9. Peptide antibiotics produced by *B. subtilis* and *B. licheniformis*

Name	*Organism:* B. subt. B. lich.	*Antibiotic spectrum*	*Molecular weight*	*Amino Acids**		*Other Components*	*References*
				D	L		
Bacillomycins		Fungi, yeasts					
Bacillomycin	*B. subt.*	Fungi, yeasts	~960	Asp, Glu, Ser, Thr, Tyr			144–147
Bacillomycin A (Fungocin)	*B. subt.*	Fungi, yeasts		Asp, Glu, Ser, Thr, Tyr (Ala, Leu, Phe, Val)			144, 146, 148
Bacillomycin B	*B. subt.*	Fungi, yeasts		Asp, Glu, Leu, Pro, Tyr			148, 149
Bacillomycin C	*B. subt.*	Fungi, yeasts		Asp, Glu, Leu, Val, Tyr			148, 150
Bacillomycin R	*B. subt.*	Fungi, yeasts		Asp, Glu, Ala, Leu, Phe, Ser, Val			148, 151
Eumycin	*B. subt.*	Fungi, yeasts		Unknown			148, 152
Toximycin	*B. subt.*	Fungi, yeasts		Unknown			148, 153
Aspergillus factor	*B. subt.*	Fungi, yeasts		Unknown			148, 154
Unnamed	*B. subt.*	Fungi, yeasts		Asp, Glu, Ala, Nval, Ser, Thr, Tyr, Phe, 2 unknown			155
Bacilysin	*B. subt.*	Gram-neg.		Ala, Leu, Phe, Tyr			156
Bacitracins							
Bacitracin A	*B. lich.*	Gram-pos.	1,400	Asp, Glu,	Ile, Leu, Cys,		157–170
	B. subt.	Gram-pos.		Phe, Orn	His, Lys, Asp		
Bacitracin F	*B. lich.*	Inactive	1,400	Asp, Glu,	Ile, Leu, His	2-Isovalerylthiazole-4-carboxylic acid	166, 171, 172
	B. subt.			Phe, Orn	Lys, Asp		

Bacitracins A, B, C, D, E, F_1, F_2, F_3, G	*B. lich.* *B. subt.*	Gram-pos. Gram-pos.		Cys, Orn, Asp, Glu, Lys, His, Leu/Ile, Phe, Val? Gly?		172, 173
Fluvomycin (Efsiomycin, Vivicil, Riomycin)	*B. subt.*	Gram-neg., fungi, yeasts		unknown		174, 175
Fungistatin	*B. subt.*	Fungi, yeasts	2,400	Asp, Lys, Ser, Thr, Pro, Ala, Ile, Val, Try, Tyr	Unidentified acid	176–178
Licheniformins Licheniformin A, B, C	*B. lich.*	Gram-pos., acid fast bact.	3,800–4,800	Arg, Asp, Gly, Lys, Phe, Pro, Ser, Val		179–181
Mycobacillin	*B. subt.*	Fungi, yeasts	~1,800	Asp, Glu | Ala, Asp, Leu | Pro, Ser, Tyr		182, 183
Mycosubtilin	*B. subt.*	Fungi		Asp, Tyr, Pro		184
Rhizobacidin	*B. subt.*	Gram-pos.		Unknown		185
Subtenolin	*B. subt.*	Gram-pos., gram-neg.		Unknown		186–188
Subtilins Subtilin A, B, C	*B. subt.*	Gram-pos.	3,420-7,000	Gly, Ala, Val, Lys, Leu, Glu, Pro, Ile, Phe, Try, β-Methyllanthionine, Lanthionine		189–192

* Whenever the configuration of the constituent amino acids has been determined, the D- and L-forms are separated by a vertical line.

tyrothricin may be used with caution in body cavities. Tyrothricin has been used in irrigating paranasal sinus cavities, but there is the risk of chemical meningitis. Dermatological sensitization is rarely observed in topical application. Gramicidin S has the same application. It was effectively used in Russia during World War II in the treatment of infected wounds[74,136]. Anosmia and parosmia may result from intranasal use. The incidence of dermatological sensitization following topical application is relatively low.

PEPTIDE ANTIBIOTICS PRODUCED BY *B. Subtilis* AND *B. Licheniformis*

General Considerations

This group of peptide antibiotics (*Table 1.9*) acts mainly against fungi, yeast and gram-positive bacteria. Only fluvomycin is stated to be active also against gram-negative bacteria. Most of these antibiotics, although isolated many years ago, are known only in their qualitative composition. They seem to contain only common amino acids in the D- and L-form, except the subtilins, in which the amino acids lanthionine and β-methyllanthionine have been found. Intensive attempts have been made to elucidate the structures of bacitracin A, bacitracin F and mycobacillin (*Figure 1.2*).

The original bacitracin-producing organism was identified as a strain of *B. subtilis*, but subsequently it was more accurately classified as a variant of *B. licheniformis*. Recently, however, a bacitracin-producing strain of *B. subtilis* was reported, and both organisms are therefore mentioned in *Table 1.9*.

The only clinically used members of this group are the bacitracins. Bacitracin is further used in veterinary medicine and as a supplement to the diets of animals, particularly farm animals[143]. The subtilins are used in the food industry as preservatives.

Bacitracin

Discovery and nomenclature

In 1945, a new antibiotic was reported[157]; it was produced by a gram-positive sporulating bacillus of the *B. subtilis* group and isolated from cultures of contaminated tissue removed from a fracture of the tibia. This strain was named after the patient 'Tracey I' and the active principle in the cell-free filtrates of broth cultures was therefore called 'Bacitracin'. The antibiotic was first produced in surface cultures[157,158] and later by submerged cultures in synthetic media[193,194]. Later the organism was classified as a strain of *B. licheniformis*[195]. In 1949 another antibiotic from a strain of *B. licheniformis*, originally called A-5, was described under the name ayfivin[159,160]. It soon became apparent that bacitracin and ayfivin were closely related in their physical, chemical and biological properties[161–163]. The name ayfivin was therefore abandoned in favour of bacitracin[163].

Chemistry

Commercial bacitracin was resolved by countercurrent distribution into a main component, called bacitracin A, and several other components designated as bacitracin B, D, E and F[172]. Later, another sample of crude

bacitracin was fractionated by the same method into at least 10 polypeptides, characterized as bacitracin A, A', B, C, D, E, F_1, F_2, F_3 and G[173]. These different components all contain cysteine, ornithine, lysine, histidine, aspartic acid, glutamic acid, leucine and/or isoleucine and phenylalanine. They differ, however, in the additional components found after acid hydrolysis and in their ultra-violet spectra[173].

Best known are the major component bacitracin A, and bacitracin F, an inactivation product of bacitracin A, through the work of Craig and his co-workers, Abraham and Newton and, to a lesser degree, Porath. The molecular weight of bacitracin A was shown by different methods to be about 1,500[196–198]. Total and partial hydrolysis gave evidence of the presence of three L-isoleucine molecules and one each of L-leucine, L-cysteine, L-histidine, L-lysine, L-aspartic acid, D-phenylalanine, D-ornithine, D-aspartic acid, D-glutamic acid and ammonia in the molecule.

After partial hydrolysis of bacitracin A[199–202] and dinitrophenylated bacitracin A,[203] followed by fractionation of the resulting peptide mixtures by countercurrent distribution, paper-chromatography, electrophoresis and ultimate sequential studies, part of the structure was proposed (*Figure 1.3*).

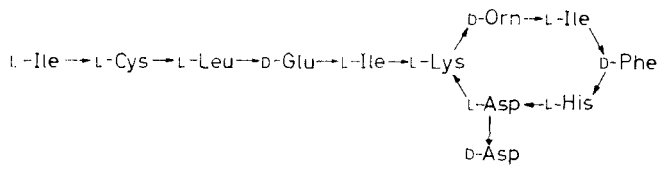


Figure 1.3. Amino acid sequence of bacitracin A

This structure, although consistent with many facts, did not explain the peculiar properties of the *N*-terminal isoleucine and the negative test for —SH groups. Further experiments led to the conclusion that a thiazoline ring is formed between isoleucine and cysteine; this was later proved to be correct. All these results led to the formula for bacitracin A which is now generally accepted[166–168] (*Figure 1.4*).

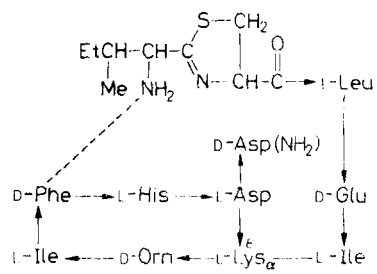


Figure 1.4. Structure of bacitracin A

Several observations indicate that the amino group of the *N*-terminal isoleucine is not entirely free and the dotted line marks a bond not yet clearly interpreted. One of the most characteristic features of the bacitracins is the limited pH range within which these antibiotics retain their activity. The slow inactivation of bacitracin at or above neutral pH was observed

very early and several proposals have been made for the structure of the resulting inactive bacitracin F. Konigsberg and Craig[171] were able to demonstrate that bacitracin F is derived from A by oxidation of the *N*-terminal amino group of the isoleucine to form a ketone, and of the thiazoline ring to a thiazole (*Figure 1.5*).

Figure 1.5. Oxidation of bacitracin A

Bacitracin B has been purified by countercurrent distribution, but it seems that this substance also is slowly transformed. Molecular weight determination and hydrolysis have given a value for a peptide containing 13 amino acids, consisting of all the amino acids of bacitracin A and an additional valine. Although some partial sequences are known, the final structure has yet to be determined[202]. Whether the other members of the bacitracin group are real entities or transformation products remains to be seen.

Synthetic approaches

Some cysteine-peptides, similar to the *N*-terminal part of bacitracin A, have been synthesized[170]. This has been done mainly to confirm certain parts of the proposed structures by synthesis.

Antimicrobial activity

Bacitracin is mainly bactericidal *in vitro* against gram-positive bacteria[157,195], including haemolytic and non-haemolytic streptococci, staphylococci, pneumococci, corynebacteria and spirochetes[204–206]. Although bacitracin has no significant effect on the majority of gram-negative bacilli, Evans[207] found that strains of *H. influenzae*, type b, from cases of meningitis were completely inhibited by bacitracin at concentrations of 0·06 to 0·6 μg/ml.

Pure bacitracin has been assigned a potency of 64·5 units/mg[208], but this rapidly declines to a level of 40–45 μg/mg. Susceptible organisms are generally inhibited by 0·05 to 2 μg/ml. (*Table 1.7*).

Bacitracin was found to be synergistic in combination with penicillin, streptomycin, or neomycin, and could, in certain limited circumstances, be antagonized by tetracycline or chloramphenicol[209–212].

Resistance to bacitracin does not emerge rapidly in originally susceptible strains[212]. The thermal and storage stability of bacitracin is enhanced by the presence of an equimolar concentration of Zn^{2+} and the antibacterial action is potentiated by an excess[213] of Zn^{2+}. It has been suggested that Zn^{2+} is also associated with the mechanism of antibacterial action[214]. No chemical assay methods are yet available for bacitracin. For the determination of potency the cylinder plate method with *Micrococcus flavus* (ATCC 10 240) as test organism is used[111].

Clinical use

Bacitracin has been used effectively in combination with other antibiotics for serious staphylococcic infections against which a single agent has not

been effective. Like the other polypeptide antibiotics, bacitracin has nephrotoxic properties, which limit its application. Although this effect is transitory in the majority of cases, systemic use of bacitracin is only recommended for treating infections due to organisms resisting safer antibiotics or chemotherapeutic agents (*Table 1.8*).

The recommended dose is 10,000 to 20,000 units given intramuscularly every 6 hours and accompanied by careful studies of the renal function[215–218]. In this way bacitracin is absorbed readily from the site of injection although pain and some injury may be observed there. It has been used in the treatment of staphylococcal bacteremias, osteomyelitis, bacterial endocarditis, and in the control of urinary tract infections either alone or in combination with other drugs[219,220]. Local injection into circumscribed areas, such as furuncles and abscesses, has also been successful. Orally, bacitracin has been used either alone or in combination with other antibiotics as an intestinal antiseptic[221], for example in the pre-operative sterilization of the bowel. Because of the poor absorption from the gastro-intestinal tract, no special precautions are necessary.

Bacitracin is mainly used for topical application. A great variety of ointments, solutions, troches and tablets are available for local application. For broadening of the antimicrobial spectrum it is very often combined with other antibiotics such as neomycin, penicillin, tyrothricin and polymixin. Topical administration is used against infections of the skin, eye, ear and throat, with little incidence of sensitization. Good results have been obtained in the prophylactic and active treatment of infected burns[222,223]. Inhalation has been reported for susceptible respiratory tract infections. Bacitracin appears to have some antihelminthic activity[224].

POLYPEPTIDE ANTIBIOTICS PRODUCED BY OTHER STRAINS OF BACILLI

General Considerations

In this section the polypeptide antibiotics produced by strains of bacilli other than *B. subtilis*, *B. brevis* and *B. licheniformis* are discussed (*Table 1.10*). Whereas alvein has been characterized by its components, the antibiotics

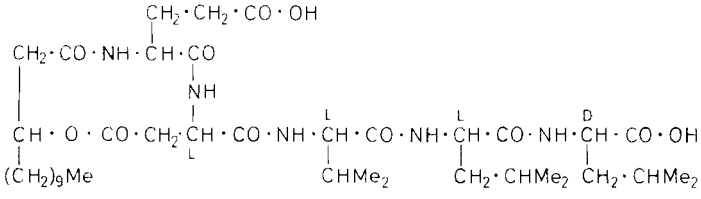


Figure 1.6. Proposed structure of esperin

cerein, the laterosporins, pumilin, tetain and toxymycin are only thought to be peptides, although nothing is known about their composition. For esperin, an acidic peptide isolated from *B. mesentericus*, a structure has been proposed by Ito and Ogawa (*Figure 1.6*) in 1959. The structure of micrococcin P is complicated and contains a number of unusual components; despite considerable effort to determine it, the correct structure is not yet known.

Table 1.10. Peptide antibiotics produced by other species of bacilli

Name	*Organism*	*Antibiotic spectrum*	*Mol. wt.*	*Amino acids**		*Other components*	*References*
				D	L		
Alvein	*B. alvei*	Gram-pos. bacteria Gram-neg. (less)		Ala, Arg, Cys, Leu, Lys, Ser, Thr, Val			225
Cerein	*B. cereus*	Gram-pos. bacteria Gram-neg. bacteria		Unknown			226
Circulins							
Circulin A	*B. circulans*	Gram-neg. bacteria	~1,200	Leu	Dab, Ile, Thr	(+)-6-methyloctanoic acid	227–236
Circulin B	*B. circulans*	Gram-neg. bacteria	~1,200	Leu	Dab, Ile, Thr	(+)-6-methyloctanoic acid	227–236
Colistins							
Colistin A (= Polymyxin E_1)	*B. colistinus*	Gram-neg. bacteria	~1,200	Leu	Dab, Leu, Thr	(+)-6-methyloctanoic acid	237–254
Colistin B (= Polymyxin E_2)	*B. colistinus*	Gram-neg. bacteria	~1,200	Leu	Dab, Leu, Thr	6-methylheptanoic acid	237–254
Colistin C	*B. colistinus*	Gram-neg. bacteria	~1,200				
Esperin	*B. mesentericus*		781	Leu	Asp, Glu, Leu, Val	β-hydroxytridecanoic acid	255
Laterosporins							
Laterosporin A, B	*B. laterosporus*	Gram-pos., gram-neg.		Unknown			256, 257
Micrococcin P (Micrococcin)	*B. pumilus*	Gram-pos.	2,170–2,720	2-(1-amino-2-isobutyl) thiazole-4-carboxylic acid, Thr		Propionic acid, 2-propionyl-thiazole-4-carboxylic acid	258–261
Polymyxins							
Polymyxin A	*B. polymyxa*	Gram-neg. bacteria	1,200	Leu, Dab?	Dab, Thr	(+)-6-methyloctanoic acid	262–266
Polymyxin B_1	*B. polymyxa*	Gram-neg. bacteria	1,200	Phe	Dab, Leu, Thr	(+)-6-methyloctanoic acid	267–278
Polymyxin B_2	*B. polymyxa*	Gram-neg. bacteria	1,200	Phe	Dab, Leu, Thr	6-methylheptanoic acid	270, 271, 279
Polymyxin C	*B. polymyxa*	Gram-neg. bacteria	1,200	Phe?, Dab?	Dab, Thr	(+)-6-methyloctanoic acid	280
Polymyxin D	*B. polymyxa*	Gram-neg. bacteria	1,200	Leu, Ser	Dab, Thr	(+)-6-methyloctanoic acid	263
Polymyxin E_1 (= Colistin A)	*B. polymyxa*	Gram-neg. bacteria	1,200	Leu	Dab, Leu, Thr	(+)-6-methyloctanoic acid	252–254, 280
Polymyxin E_2 (= Colistin B)	*B. polymyxa*	Gram-neg. bacteria	1,200	Leu	Dab, Leu, Thr	6-methylheptanoic acid	252–254, 280
Polymyxin M	*B. polymyxa*	Gram-neg. bacteria	1,200	Leu	Dab, Thr	(+)-6-methyloctanoic acid	281–283
Polypeptin	*B. krzemieniewski*	Gram-neg. bacteria	1,145	Phe, Val	Dab, Ile, Leu, Thr		229, 284–287
Pumilin	*B. pumilus*	Gram-pos. bacteria		Unknown		Unknown	288
Tetain	*B. pumilus*	Gram-pos., gram-neg.		Unknown		Unknown	289–291
Toxymycin	*Bacillus sp.*	Fungi		Unknown		Unknown	292

* Whenever the configuration of the constituent amino acids has been determined, the D- and L-forms are separated by a vertical line.

The important and clinically used antibiotics of this class are the colistins and the polymyxins, which form a group of closely related substances. Isolated more than 15 years ago and clinically used for the last 10 years, their structures have been revealed only recently. They are characterized by a high and specific activity towards gram-negative bacteria, by their basic nature and by a fatty acid component. Because of their close chemical and biological relationship they may now be considered as members of the polymyxins. Although they are the most active inhibitors of the growth of gram-negative bacteria, they have so far been considered as 'minor antibiotics' and their toxicity has limited their application. However, the increase of serious infections with gram-negative bacteria and the growing resistance to broad spectrum antibiotics have recently attracted more and more interest to this group of antibiotics.

Several comprehensive reviews have appeared[293–295] and the ones by Jawetz[294] in 1956 and Schwartz[295] in 1964 give valuable information about their clinical application. The main emphasis here will therefore be on the chemistry of these compounds. The recent structural elucidations and the syntheses have cleared much of the confusion existing in this group of antibiotics.

The Polymyxin Group

Discovery and nomenclature

The polymyxins—The discovery of antibiotic substances from various strains of *Bacillus polymyxa* was announced within four months in 1947 by three independent groups. Benedict and Langlykke[296] noted the antibacterial activity of *B. polymyxa* on agar and described a medium for obtaining culture filtrates active against *Brucella bronchiseptica*. Stansly, Shepard and White[297] reported on the production, isolation and purification of an antibiotic substance from *B. polymyxa*, which was called 'polymyxin'. In this and following papers[298–301] they described some physical, chemical and biological properties which distinguished polymyxin from certain known antibiotics. Finally Ainsworth, Brown and Brownlee[262] published a communication about an antibiotic principle isolated from *Bacillus aerosporus Greer* under the name of aerosporin and later reported on its chemotherapeutic and pharmacological properties[302].

It soon became apparent that the three groups were dealing with closely related substances when it was possible to prove the identity of *B. aerosporus* and *B. polymyxa*[303,304]. All three antibiotics acted selectively against gram-negative bacteria and were nephrotoxic. Several investigators, especially Jones[305], recognized that these organisms produce a whole family of antibiotics which are chemically closely related polypeptides and biologically practically indistinguishable. In an attempt to clarify the situation, the groups of investigators collaborated in comparative chemical and biological studies, and their results were presented at a symposium at the New York Academy of Sciences in May 1948[306]. 'Polymyxin' was accepted as generic name for all the related antibiotics produced by *B. polymyxa*[304]. Alphabetical suffixes were adopted to distinguish the antibiotics on the basis of their amino acid composition[304,305,307]. Aerosporin[262] was renamed polymyxin A

and the polymyxin of Stansly, Shepard and White[297] became polymyxin D. Three other active substances isolated from different strains of *B. polymyxa*[280,304,305] were designated as polymyxin B, C and E. Polymyxin B was later separated by countercurrent distribution into two different components, polymyxin B_1 and B_2[271]; similarly polymyxin E could be fractionated into polymyxin E_1 and E_2[254].

Another strain of *B. polymyxa*, isolated from soil in Moscow in 1958, produced a different type of polymyxin, called polymyxin M[281,282].

The colistins—Although the history of the colistins is less complex, there is still some confusion about the name. In 1950 the isolation of an antibiotic from cultures of *Aerobacillus colistinus* was described under the name of colistin[237,238]. The culture filtrates had a significant inhibitory activity against many gram-negative bacteria. *Aerobacillus colistinus* and *B. polymyxa* are taxonomically related to each other. This antibiotic is also known under the names Colimycine, Colomycin and Coly-Mycin, but it should not be confused with the Russian antibiotic colymycin[308] which is related to neomycin. Commercial colistin was separated by paper-chromatography[243] and by countercurrent distribution[250] into three components which were designated colistin A, B and C. Colistin A and B have much stronger antibacterial activity than C. Sometimes commercial material does not contain colistin C[250]. Very recently it was shown that colistin A and colistin B are identical to polymyxin E_1 and polymyxin E_2[252–254].

The circulins—In May 1948, Murray and Tetrault[227] announced the discovery of an antibiotic, which was more active against gram-negative than gram-positive organisms and which resembled the polymyxin-type of antibiotics. They were later able to identify[228] the organism as a non-haemolytic strain of *Bacillus circulans* and therefore suggested the name 'circulin'. Circulin was subsequently shown by chromatography to consist of two major components called circulin A and circulin B, besides possibly some other components[230,231].

Polypeptin—The antibiotic polypeptin was isolated in 1948 by McLeod[284] from a culture of *Bacillus krzemieniewski*, a mucoid variant of *B. circulans*, and given the name of circulin. It is primarily active against gram-negative bacteria, but is also inhibitory to many gram-positive bacteria and fungi. It was soon obvious that this substance differed from the antibiotic circulin described by Murray and Tetrault[227,228]. By mutual agreement between the groups involved[229] the name circulin was reserved for the antibiotic of Murray and Tetrault[227,228], while the antibiotic of McLeod[284] was renamed polypeptin.

Chemistry

The polymyxins—The members of the polymyxin group have very similar chemical and physical properties. They are all cyclic basic polypeptides containing characteristic constituents such as α,γ-diaminobutyric acid, L-threonine and a fatty acid. They differ by the presence or absence of the additional amino acids D-leucine, L-leucine, L-isoleucine, D-phenylalanine, D-valine and D-serine, as well as by the nature of the fatty acid (*Table 1.11*). Their molecular weights are all in the region of 1,200. Although several structural features had been established very early[269,270,306] the exact

Table 1.11. Comparative amino acid and fatty acid composition of the polymyxins*

Antibiotic	*Dab*	L-*Thr*	L-*Leu*	D-*Leu*	L-*Ile*	D-*Phe*	D-*Ser*	D-*Val*	*Fatty acid*	*References*
Polymyxin A	+	+	—	+	—	—	—	—	MOA	265
Polymyxin B_1	6	2	1	—	—	1	—	—	MOA	267, 270–272, 275–278
Polymyxin B_2	6	2	1	—	—	1	—	—	IOA	271, 309
Polymyxin C	+	+	—	—	—	+	—	—	MOA	280
Polymyxin D	5	3	—	1	—	—	1	—	MOA	263, 280
Polymyxin M	6	3	—	1	—	—	—	—	MOA	263, 317
Colistin A (polymyxin E_1)	6	2	1	1	—	—	—	—	MOA	248, 250, 251, 253, 254, 267, 277, 309, 323
Colistin B (polymyxin E_2)	6	2	1	1	—	—	—	—	IOA	248, 250, 253, 254, 309, 324
Circulin A	6	2	—	1	1	—	—	—	MOA	232–235, 325
Circulin B	6	2	—	1	1	—	—	—	MOA	232–235
Polypeptin	3	1	2	—	1	1	—	1	Unknown	287

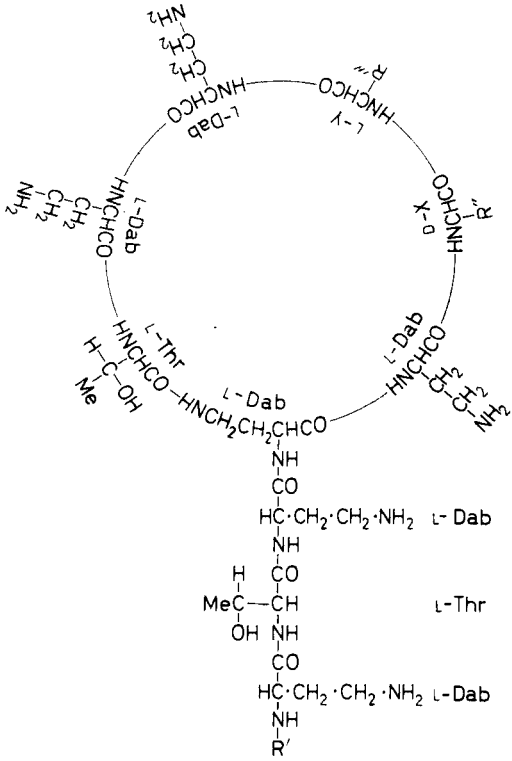
* The figures give the number of amino acid residues per molecule. The + and — signs indicate the presence or absence of the amino acids. MOA = (+)-6-methyloctanoic acid; IOA = Iso-octanoic acid (6-methylheptanoic acid).

chemical study began in 1954, when Hausmann and Craig succeeded in separating commercial polymyxin B into the homogeneous fractions B_1 and B_2 by countercurrent distribution[271]. On hydrolysis both gave α,γ-diaminobutyric acid, threonine, leucine and phenylalanine. From the hydrolysate of B_1, (+)-6-methyloctanoic acid[270,271] was isolated whereas B_2 yielded a different, optically inactive fatty acid, which was later shown by Wilkinson and Lowe to be 6-methylheptanoic acid[253,309].

The molecular weight of polymyxin B_1 was shown by the method of partial substitution[310] to be 1,150 ± 10 per cent. Quantitative amino acid analysis yielded the amino acids α,γ-diaminobutyric acid, L-threonine, D-phenylalanine and L-leucine in the molar proportions 6:2:1:1. Because no free α-carboxyl- and no free α-amino groups could be detected, polymyxin B_1 had to be of a cyclic nature[272]. Neither pepsin nor trypsin was found to attack the molecule and therefore partial hydrolysis and separation of the fragments was used for the elucidation of the structure. Of the 14 fragments isolated and identified, seven key peptides were necessary for proposing four tentative structures for polymyxin B_1[272]. Two were formulated with a ring of eight amino acids having a side-chain attached either in the α- or γ-position of the branching α,γ-diaminobutyric acid (8α, 8γ) and two with seven amino acids in the ring and a side-chain equally attached in the α- or γ-position (7α, 7γ)[272,311]. Biserte and Dautrevaux, who confirmed these results later[275], found in addition that the α,γ-diaminobutyric acid residue adjacent to the fatty acid had the D-configuration. Swiss workers subsequently synthesized all of the four proposed structures, but found that none of them was identical to the natural polymyxin B_1[311–316]. Recently, however, Japanese workers[267,276] have been able to degrade polymyxin B_1 by the enzyme nagarse (subtilopeptidase A, EC 3.4.4.16, a proteinase preparation from *B. subtilis*) and to establish the structure 7α unequivocally (*Figure 1.7*). In addition they found that all the α,γ-diaminobutyric acid residues are of the L-configuration. Analogous results were also obtained by Wilkinson and Lowe[277]. Final proof of the structure of polymyxin B_1 was achieved by total synthesis by Vogler, Studer, Lanz, Lergier and Böhni[278] (*Figure 1.8*).

Recently it has been reported that polymyxin B_2 differs from polymyxin B_1 only in the replacement of the (+)-6-methyloctanoic acid by 6-methylheptanoic acid[309]. Polymyxin A[265] and C[280] are only known in their qualitative, and polymyxin D[263] and M[283,317] only in their quantitative amino acid composition (*Table 1.11*). Polymyxin M is at present being studied intensively[318–322].

The colistins—Colistin has been investigated by several authors and various tentative structures have been proposed. This is because colistin is also a mixture of chemically closely related peptides. In 1953 Japanese chemists[243] separated commercial colistin by paper-chromatography into three different components, and Morito[249] reported in 1961 on the separation of colistin into the components A_1 and A_2 by countercurrent distribution. In 1963 commercial colistin was fractionated by the same method into three components[250], which were designated A, B and C and which corresponded to those found earlier[243,245]. Colistin C was found only in small quantities and sometimes it was absent in commercial material.



	R′	R″	R‴	X	Y
Polymyxin B_1	MOA	CH_2Ph	Bu^i	Phe	Leu
Polymyxin B_2	IOA	CH_2Ph	Bu^i	Phe	Leu
Colistin A (=Polymyxin E_1)	MOA	Bu^i	Bu^i	Leu	Leu
Colistin B (=Polymyxin E_2)	IOA	Bu^i	Bu^i	Leu	Leu
Circulin A	MOA	Bu^i	Bu^s	Leu	Ile

Figure 1.7. The structures of the known members of the polymyxin group

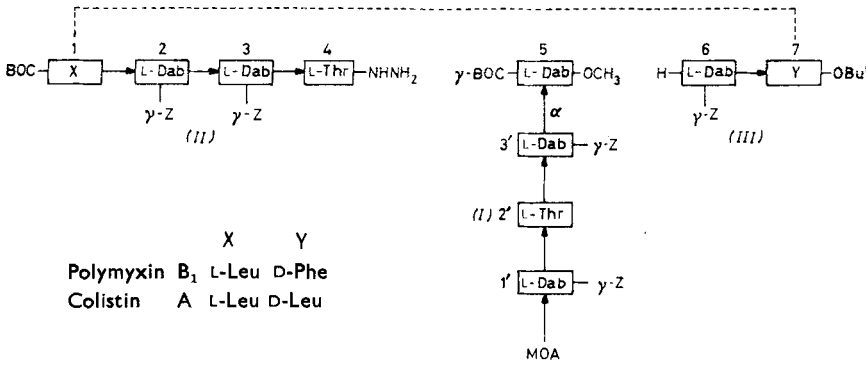


Figure 1.8. Reaction scheme for the synthesis of colistin A and polymyxin B_1. BOC = tertiary butyloxycarbonyl; Z = benzyloxycarbonyl; OBu^t = tertiary butylester; MOA = (+)-6-methyloctanoic acid

Molecular weight determination by the method of partial substitution[310] and spectrophotometric measurements of picrates gave values of about 1,360 for the hydrochlorides of both colistin A and B[250]. Quantitative amino acid analysis showed the presence of six moles of L-α,γ-diaminobutyric acid, two moles of L-threonine, one mole of L-leucine and one mole of D-leucine in both colistin A and B[248,250]. By partial acid hydrolysis as well as by enzymatic degradation with nagarse (subtilopeptidase A, EC 3.4.4.16) followed by fractionation and structural determination of the resulting peptides, a structure was proposed[251,267] for colistin A (*Figure 1.7*), which differed from those previously reported but closely resembled the structure of polymyxin B_1. By acid hydrolysis Wilkinson and Lowe[277] arrived at two possible structures, one of which was identical to that derived by Suzuki. This structure was confirmed by the total synthesis of colistin A by Vogler and Studer[323]. Thus colistin A differs from polymyxin B_1 only by the replacement of D-phenylalanine with D-leucine. By virtually the same methods, Suzuki and Fujikawa elucidated the structure of colistin B and found that it differs from colistin A only in having 6-methylheptanoic acid in place of 6-methyloctanoic acid[324].

Recently Wilkinson and Lowe reported on the fractionation of polymyxin E into two components, polymyxin E_1 and polymyxin E_2. Structural degradation revealed that colistin A is identical to polymyxin E_1, and colistin B identical to polymyxin E_2[253,254,309].

The circulins—As early as 1949, Peterson and Reineke[230] characterized circulin as its sulphate. Total hydrolysis yielded D-leucine, L-threonine and L-α,γ-diaminobutyric acid together with an optically active isomer of pelargonic acid. The existence of two components, found by Peterson and Reineke[230] was later confirmed[231] by the chromatographic separation of crude circulin into two major components, named circulin A and circulin B. In addition there was evidence for at least three other ninhydrin-positive, biologically active entities. In the hydrolysate of circulin A, L-isoleucine was found besides the amino acids previously reported[230]. Quantitative amino acid analysis[232,233] showed circulin A and B to be composed of L-α,γ-diaminobutyric acid, L-threonine, D-leucine, L-isoleucine and (+)-6-methyloctanoic acid in the molar ratio 6:2:1:1:1. After partial acid hydrolysis, fractionation and structure determination of the resulting peptides, circulin A and circulin B were formulated as cyclodecapeptides[234,235]. Very recently, however, Japanese workers[325] have revised the structure of circulin A. According to them, circulin A differs from colistin A only by a replacement of L-leucine in the latter by L-isoleucine (*Figure 1.7*).

Polypeptin—Polypeptin was purified, crystallized as its sulphate and characterized by Howell[286]. By means of countercurrent distribution, Hausmann and Craig determined the components as L-α,γ-diaminobutyric acid, L-threonine, D-valine, L-isoleucine, L-leucine and D-phenylalanine in the molar ratio 3:1:1:2:1 and an unknown fatty acid[287]. The molecular weight of the free base was found to be 1,145. The structure of polypeptin is still unknown.

Synthesis

Among the members of the polymyxin family the structures of polymyxin B_1, polymyxin B_2, colistin A (polymyxin E_1), colistin B (polymyxin E_2) and

circulin A are known to date. Of these, polymyxin B_1[278] and colistin A[323] have been synthesized. Because of the close chemical relationship of these two antibiotics (*Figure 1.7*), their syntheses followed virtually the same scheme (*Figure 1.8*).

The three key peptides were synthesized by the active ester method. Removal of the tertiary butyloxycarbonyl group in (*I*) and coupling with the azide of (*II*) led to the protected octapeptide. This was transformed into the corresponding hydrazide and condensed with (*III*) by means of the azide procedure. After removal of the *C*- and *N*-protecting groups the decapeptide was submitted to cyclization in high dilution with carbodi-imide and was then reduced and purified extensively by countercurrent distribution, precipitation over the free base and crystallization as pentaphosphate. The resulting polymyxin B_1 and colistin A were found to be identical to the corresponding natural products.

Antibacterial activity

The *in vitro* antibacterial activities of polymyxin B and colistin are almost identical[140,141,326,327]. They have a unique *in vitro* bacteriostatic and bactericidal activity against a wide spectrum of gram-negative bacteria, especially against *Aerobacter aerogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, *Haemophilus influenzae*, *Haemophilus pertussis*, *Pseudomonas aeruginosa*, and many strains of *Salmonella*, *Shigella*, *Pasteurella* and *Brucella*. Their minimum inhibitory concentration is less than 10 μg/ml., and most bacteria are inhibited by a concentration ranging from 0·05 to 2 μg/ml. Especially against *Pseudomonas aeruginosa*, the polymyxins and colistins are more effective by weight than any other antibiotic[328,329]. In an investigation of a large number of coliform bacilli isolated in a hospital in the United States[329] it was found that polymyxin and colistin were at least as effective as streptomycin, kanamycin, chloramphenicol and tetracycline against 94 per cent of *E. coli*, 86 per cent of *Klebsiella*, 93 per cent of *Pseudomonas pyocyanea* and 50 per cent of *Paracolon*. These findings applied to bactericidal as well as to bacteriostatic tests (*Table 1.7*).

Most strains of *Proteus* are unaffected[140,141,327], some strains of *Neisseria*[140] are also resistant, and gram-positive bacteria are usually not inhibited[140,141,329]. The *in vitro* activity of polymyxin and colistin against fungi is moderate[140]. Very high concentrations (20 to >250 μg/ml.) are fungistatic or fungicidal against several organisms, including *Candida albicans*, and some species of *Trichophyton* and *Microsporum*.

The therapeutic effectiveness of the polymyxins[140,329–334] and colistin[140,335] has been determined against a variety of experimental gram-negative infections, particularly in mice. In general the *in vivo* results parallel those *in vitro*. Both are effective when administered subcutaneously, intraperitoneally or intravenously, but are either inactive or only slightly active when given orally[336,337]. Significant differences in the relative effectiveness of colistin sulphate, colistin methane sulphate and polymyxin B sulphate against *K. pneumoniae* and *E. coli* infections in mice have been demonstrated[140].

In contrast to many other, primarily bacteriostatic drugs, both colistin[140,338] and polymyxin[332,339] rarely induce resistance during repeated passage of normally sensitive strains in subinhibitory concentrations.

Synergistic action of colistin and polymyxin in combination with other antibiotics *in vitro* and *in vivo* has been reported by several investigators[340–347]. In clinical application the effect of these combinations seems to be more a broadening of the antimicrobial spectrum than a synergistic interaction. Several salts and derivatives of both colistin and polymyxin have been reported. Commercially available are the sulphates and also recently the sodium sulphomethyl derivatives which are prepared by treatment of the free bases of polymyxin and colistin with formaldehyde and sodium bisulphite[348]. These derivatives have a lower toxicity than the sulphates, however, and their *in vitro* antimicrobial activity is also reduced.

No chemical methods for assaying either polymyxin or colistin are available at the present time. For the determination of potency either the cylinder plate assay using *Brucella bronchiseptica* ATCC 4617 as test organism or the nephelometric method[349] is used[111]. The current USP standards are 7,850 units/mg for polymyxin B and 20,070 units/mg for colistin.

Structure–activity relationships

It is still premature to give a clear correlation between structure and activity in the polymyxin group, because most published data on antimicrobial activity have been obtained either with impure material or with mixtures of the different individuals. Also the definition of potency differs for those antibiotics which were considered to be different up to now. But the synthesis[311–316] of the four compounds originally proposed by Hausmann with a D-α,γ-diaminobutyric acid adjacent to the fatty acid and the synthesis of polymyxin B_1[278] and colistin A[323] have given some clues in these respects (*Table 1.12*).

The antimicrobial activity *in vitro* and *in vivo* is strongly connected with the ring size. The two analogues of polymyxin B_1 with eight amino acid residues in the ring are about ten times less active than the corresponding analogues with seven amino acid residues in the ring.

The nature of the amino acids seems to be of minor importance. The presence of the D- instead of the L-α,γ-diaminobutyric acid has a significant influence only on the activity against *K. pneumoniae in vitro* and *E. coli in vivo*. The exchange in polymyxin B_1 of D-phenylalanine by D-leucine to give colistin A and of D-phenylalanine and L-leucnine by D-leucine and L-isoleucine to give circulin A seems to have little effect. Furthermore, the connection of the side-chain in the α- or γ-position of the branching α,γ-diaminobutyric acid exerts a minor influence. The antimicrobial activity is definitely connected with the basic amino groups. Acetylation and benzyloxycarbonylation of polymyxin leads to completely inactive material, and the sodium sulphomethyl derivatives[348,350,351] have a lower activity than the corresponding parent compounds[348]. The structure of the fatty acid, however, is obviously of minor influence, since colistin A and colistin B, which differ only in their fatty acid, are of nearly equal activity[250].

The whole character of the molecule seems to be responsible for the biological activity of the polymyxins. One might expect that a conformation similar to grāmicidin S is responsible, in so far as the hydrophilic N^{γ}-amino groups are aggregated on one side of the molecule and the hydrophobic side-

Table 1.12. Microbiological activity of the synthetic isomers 8γ, 8α, 7γ, 7α and colistin A in comparison with natural polymyxin B_1, standard deviation $\pm$20 per cent[311,313,316,323]

	8γ	8α	7γ	7α	*Colistin A*	B_1
Brucella bronchiseptica ATCC 4617 (*in vitro*)	900 u/mg	800 u/mg	8,250 u/mg	8,898 u/mg	4,983 u/mg	8,096 u/mg
Pseudomonas aeruginosa (*in vitro*)	322 u/mg	396 u/mg	7,088 u/mg	6,761 u/mg	—	10,420 u/mg
Escherichia coli ATCC 10'536 (*in vitro*)	241 u/mg	184 u/mg	4,681 u/mg	5,081 u/mg	5,775 u/mg	5,033 u/mg
Klebsiella pneumoniae ATCC 100'131 (*in vitro*)	—	—	1,297 u/mg	1,514 u/mg	7,381 u/mg	8,421 u/mg
Escherichia coli 1346 (LD_{50} s.c., mice) *in vivo*	7 mg/kg	7 mg/kg	2·7 mg/kg	2·0 mg/kg	—	0·7 mg/kg

chains together with the fatty acid are situated on the other side. Such a conformation would also here explain the pronounced surface activity.

Clinical use

Polymyxin B, the mixture of B_1 and B_2, and colistin, the mixture of colistin A, B and C, are the only clinically used members of this group of antibiotics. Owing to their close chemical and biological relationship they are effective in similar types of infections. They are both used in the treatment of infections of the urinary tract[294,295,352–359], bacteremia[360,361], bacterial meningitis[362,363], burns and wounds, infections[364–367], gastroenteritis[368–371], and other infections due to *E. coli*, *A. aeruginosa*, *K. pneumoniae*, *Shigella*, *H. influenzae*, and especially *Pseudomonas aeruginosa*[140,141,218,327,328,352,372–376].

Several toxic side-effects have been reported when polymyxin B and colistin are administered parenterally. Besides local irritation and pain at the site of injection in intramuscular administration, marked nephrotoxic effects are observed manifested by proteinuria, and cylindruria accompanied occasionally by an increase in white, red and epithelical cells in the urinary sediment. The neurotoxic effects of the drugs are characterized by flushing of the face, drowsiness, and a feeling of weakness and irritability. These symptoms, however, are transitory and disappear upon removal of the drug. In patients with pre-existing renal damage polymyxin and colistin should be administered in lower doses under frequent control of the renal functions. The recently available sodium sulphomethyl derivatives of polymyxin B and colistin are stated to be less toxic, yet these derivatives are also less active than their parent compounds[140,348,351,352].

Orally, both antibiotics are used for the treatment of intestinal infections due to susceptible organisms and, in combination with other drugs, for bowel sterilization prior to surgery. Owing to poor absorption from the gastro-intestinal tract, this form of application has rarely led to side-effects. Both are used either alone or in combination with other antibiotics in a variety of ointments and solutions for topical application on infected surface lesions of skin and mucuous membranes[364–366]. Allergic reactions have rarely been observed.

Provided application is careful, these antibiotics constitute a valuable tool for the treatment of a variety of infections, despite their potential toxicity (*Table 1.8*).

POLYPEPTIDE ANTIBIOTICS PRODUCED BY STREPTOMYCES

General Considerations

By far the largest and still rapidly expanding group of peptide antibiotics has been isolated from the streptomyces species (*Table 1.13*). In contrast to the antibiotics produced by other organisms, especially bacilli, these substances are characterized by their great variety of unusual components. Besides many hitherto unknown amino acids, these compounds contain heterocycles, sugars, amino sugars, hydroxy acids, fatty acids and some contain iron. Ester bonds have been reported for some of these antibiotics. Ilamycin represents the only antibiotic yet found consisting only of L-amino acids. Like several bacilli species many streptomyces produce whole groups

of closely related substances. These differ usually only by one single component, while the rest of the molecule is unchanged.

Structures (*Figure 1.9*) have been proposed for etamycin, echinomycin, albomycin, ilamycin, telomycin, viomycin, valinomycin and for some members of the streptothricins, the actinomycins and the vernamycins. Syntheses have only been achieved for valinomycin and some members of the actinomycins.

Besides the actinomycins, which are not included in this review, amphomycin and viomycin are at the present time the only clinically used members of this group.

Amphomycin

Discovery and nomenclature

In 1953 Heinemann, Kaplan, Muir and Hooper isolated a crystalline antibiotic substance from several streptomyces strains. The strain selected for the production of the antibiotic was isolated from soil collected near Syracuse, New York, and called *Streptomyces canus*. Because of the amphoteric properties, the antibiotic was named amphomycin[382]. It seems that amphomycin is closely related if not identical to crystallomycin[472].

Chemistry

Amphomycin is a highly surface active acidic polypeptide with an isoelectric point of 3·5 to 3·6[382]. One free amino group[382,473] and three to four acidic groups have been determined[382]. It crystallized as the calcium salt. Total acid hydrolysis and separation of the components by combined electrophoresis and paper-chromatography showed glycine, proline, valine, aspartic acid, an unknown monoaminomonocarboxylic acid, $C_6H_{11}NO_2$, and an unknown diaminomonocarboxylic acid, $C_7H_{14}N_2O_2$, to be present[472,474]. Aside from these amino acids, the hydrolysate contained a nitrogen-free saturated aliphatic acid[472]. This composition suggested that amphomycin is very closely related to and perhaps identical to the antibiotic crystallomycin[472]. Hydrolysis of amphomycin in the presence of cation-exchange resins led to a mixture of peptides, which have not been identified[475]. The structure of amphomycin is unknown.

Antimicrobial activity

Amphomycin is mainly active against gram-positive bacteria, with little or no activity against gram-negative bacteria or yeast[382]. As its sodium salt, amphomycin has definite therapeutic effects against experimental *Tr. gambiense* and *Tr. rhodesiense* infections[476] in mice and a curative value for fatal avian spirochetosis in chicks[477]. It has successfully protected mice infected by intraperitoneal inoculation with 100 LD_{50} of *Diplococcus pneumoniae*[382]. It has been reported that the antibacterial activity of crystallomycin and amphomycin is lower in the presence of phosphates, but is completely restored after their removal[478] (*Table 1.7*).

Clinical use

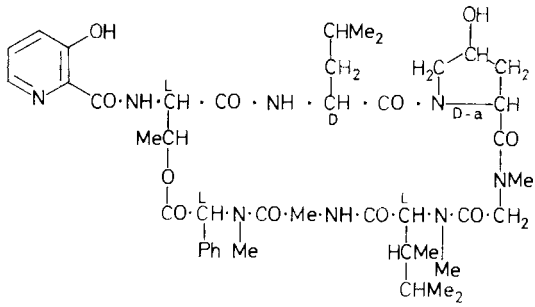
The calcium salt of amphomycin has a lethal dose (LD_{50}) (*Table 1.8*) of 120 mg/kg when given intravenously and haemolysis has been observed

Table 1.13. Polypeptide antibiotics produced by streptomyces

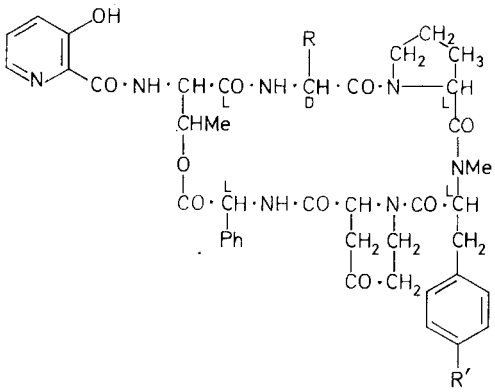
Name	*Organism*	*Antibiotic spectrum*	*Mol. wt.* (*Est.*)	*Amino acids* D	*Amino acids* L	*Other components*	*References*
Actinomycin	*S. antibioticus*	Gram-pos. bacteria, antineoplastic	1,300	alle, Val	Sar, Me-Val, Pro, Thr, Me-Ile, Me-Ala, Hypro	Actinocinin	9, 377–379
Alazopeptin	*S. griseoplanus*	Antineoplastic	~400		Ala, 6-diazo-5-oxo-amino-hexanoic acid		380, 381
Amphomycin (crystallomycin)	*S. canus*	Gram-pos. bacteria		Asp, Gly, Pro, Val, 2 unidentified		Unidentified aliphatic acid	382–384
Antimycins (Antimycin, Blastmycin Phyllomycin, Virosin)	*S. sp.*	Fungi			Thr, 3-aminosalicylic acid	α-n-butyryl-β, γ-hydroxy-pentanoic acid, α-n-hexyl-β, γ-dihydroxy-pentanoic acid, formic acid	385–389
Aspartocin	*S. griseus*, *S. violaceus*	Gram-pos. bacteria		α-pipecolic acid	Asp, α, β-Dab, β-Me-Asp, Gly, Pro, Val	(+)-12- and 13-methyl-3-tetradecenoic acid	390–392
Bottromycin	*S. bottropensis*	Gram-pos. bacteria, acid-fast bacteria	743	Gly, Val, β-(2-thiazole)-β-Ala, α-amino-β-phenylbutyric acid			393–396
Capreomycins Capreomycin I, II	*S. capreolus*	Antimycobacterial activity		Ala, α, β-diaminopropionic acid, β-Lys, α-(2-imino-hexahydro-4-pyrimidyl)-glycine, Ser			397
Cinnamycin	*S. cinnamoneus*	Gram-pos. bacteria, acid-fast bacteria		Arg, Asp, Glu, Phe, Lanthionine, β-methyl-lanthionine, Pro, Val			398, 399
Danomycin	*S. albaduncus*	Gram-pos. bacteria		Asp, Glu, Gly, Ile, Leu, Pro, Ser, Thr		Fe	400
Duramycin	*S. cinnamoneus*	Gram-pos. bacteria, fungi, yeast		Asp. Glu, Gly, Phe, Pro, Val, Lanthionine, β-methyl-lanthionine			401, 402
Etamycin (Virodogrisein)	*S. lavendulae* *S. griseus*	Gram-pos. bacteria	800	Leu, a Hypro	Ala, β-*N*-dimethylleu, α-Phenylsar, Sar, Thr	3-hydroxypicolinic acid	403–406
Ilamycins (Rufomycin)	*S. islandicus*	Acid-fast bacteria	1,100		Ala, Leu, Me-Leu, γ-formyl-methyl-norvaline, 3-nitro-4-hydroxy-Phe, Try, 2-amino-4-hexenoic acid		407–409
Matamycin	*S. matensis*	Gram-pos. bacteria, gram-neg. bacteria		Ala, Arg(?), Cys, Gly, Ser			410, 411
Netropsin (Congocidin, T 1384 Distamycin)	*S. netropsis* *S. sp.*	Fungi	~500	β-amino-propionic acid 1-methyl-4-aminopyrrole-2-carboxylic acid 1-methyl-4-aminopyrrole-2-carboxylic acid		Formic acid Guanidinoacetic acid	412–415

Pyridomycin	*S. pyridomyceticus*	Antimycobacterial activity	520	Gly, Thr		3-hydroxypicolinic acid, β-picoline	416
Quinoxalines	*S. echinatus*	Gram-pos. bacteria	850	Ser	Ala, *N*, *N'*-dimethylcystine, Me-Val	Quinoxaline-2-carboxylic acid	417–425
(Echinomycin, Quinomycin, Actinoleukin, Levomycin, Triostin, X-948)	*S. aureus*	Gram-neg. bacteria Acid-fast bacteria Antineoplastic			Me-Ile, *N*-β-dimethylleucine		
Saramycetin	*S. saraceticus*	Fungi, yeasts	14,000	Asp, Cys, Gly, Pro, Thr			
Sideromycins	*S. griseoluteus*	Gram-pos. bacteria	~1,000		Glu, Ser, Pro, *N*-hydroxyornithine,	Fe, succinic acid,	426
(Ferrimycin, Grisein, Albomycin)	*S. subtropicus*				δ-aminovaleric acid, unidentified	1-amino-5-hydroxylaminopentane, cadaverine, 3-methyluracil	427–434
Stendomycin	*S. sp.*	Fungi	1,850	Ala, Ile, Leu, Gly, Pro, Ser, Thr, Val, 2 unidentified			435
Streptothricins (Geomycin, Mycothricins, Pleocidin, Racemomycin, Roseothricin, Streptin, Streptolin, Streptothricin)	*S. lavendulae* *S. racemochromogenus* *S. xantophaeus*	Gram-pos. bacteria Gram-neg. bacteria Fungi, yeasts	~500		β-Lys, streptolidine	D-gulosamine, other sugars	436–448
Telomycin	*S. sp.*	Gram-pos. bacteria	~1,000		*cis*-3-Hypro, trans-3-Hypro, Ala, Asp β-Hyleu, Ser, a Thr, Try, β-Me-Try		449, 450
Thiostreptons (Thiactin, Bryamycin Siomycin)	*S. azureus* *S. hawaiiensis*	Gram-pos. bacteria	1,650	Cys	Ala, Ile, Thr Thiostreptoic acid, thiostreptine		451–455
Valinomycin	*S. fulvissimus*	Acid-fast bacteria, fungi, yeasts	1,150	Val	Val	D-α-hydroxyisovaleric acid, lactic acid	456, 457
Vernamycins	*S. loidensis*	Gram-pos. bacteria	~700	Amino-butyric acid, Ala,	Asp, Phe, Pro, Thr, phenylglycine,	3-hydroxypicolinic acid	458–466
(Doricin, Mikamycin B Ostreogrycin, PA 114 B Staphylomycin Streptogramin)	*S. virginiae* *S. ostreogryceus* *S. matakaensis* *S. graminofaciens*	Gram-neg. bacteria Acid-fast bacteria			Mo-Phe, *N*-methyl-*p*-dimethylaminophenylalanine, *N*-methyl-*p*-methylaminophenylalanine, 4-oxopipecolic acid, Sar		
Viomycin	*S. floridae* *S. vinaceus* *S. puniceus*	Gram-pos. bacteria Gram-neg. bacteria Acid-fast bacteria	475		β-Lys, α, β-diaminopropionic acid, Ser	Viomycidine	467–469 470, 471

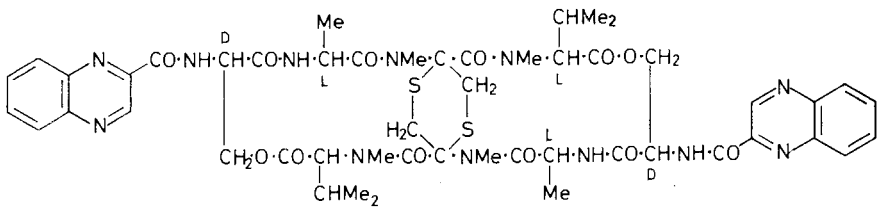
* Whenever the configuration of the constituent amino acids has been determined, the D- and L-forms are separated by a vertical line.



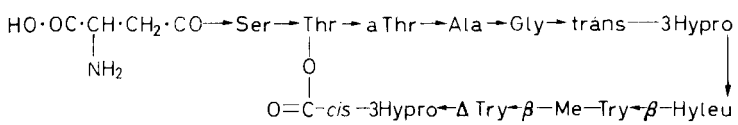
Etamycin



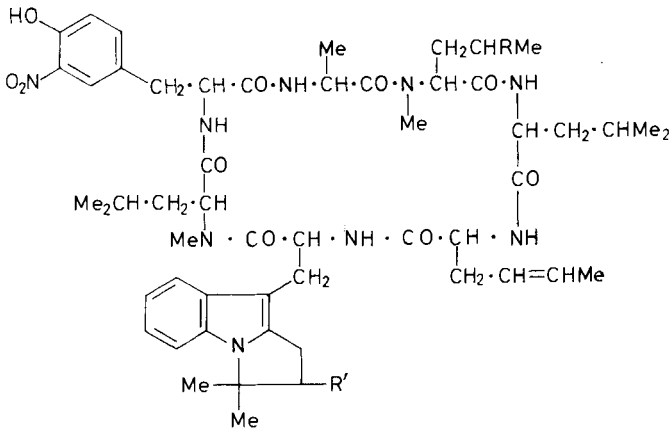
Staphylomycin R = Et, R′ = H
Vernamycin B_α (Ostreogrycin B) R = Et, R′ = NMe_2
Vernamycin B_β (Ostreogrycin B_2) R = Et, R′ = NHMe
Vernamycin B_γ (Ostreogrycin B_1) R = Me, R′ = NMe_2
Vernamycin B_δ R = Me, R′ = H



Echinomycin

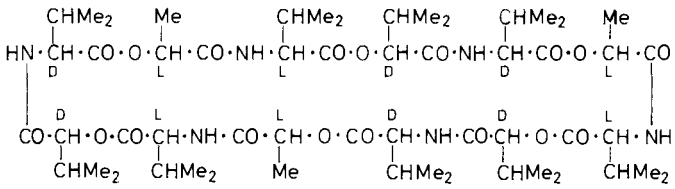


Telomycin

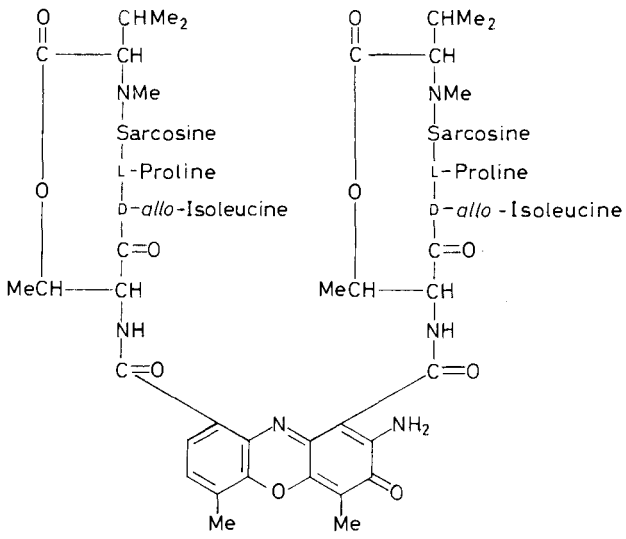


Ilamycins (Rufomycins)

Ilamycin	R=CHO,	R′=OH
Ilamycin B_1	R=Me,	R′=H
Ilamycin B_2	R=Me,	R′=OH



Valinomycin



Actinomycin C_3

Figure 1.9. Structures of antibiotics produced by streptomyces

after parenteral application. Therefore this antibiotic is mainly confined to topical application. It is available in ointments alone or in combination with other local antibiotics for the treatment of lesions infected with staphylococci, streptococci and other gram-positive organisms.

Viomycin

Discovery and nomenclature

Viomycin was obtained independently and simultaneously by two different laboratories[467,468] from actinomycetes designated as *Streptomyces puniceus*[467] and *Streptomyces floridae*[468]. Later another antibiotic with pronounced antituberculosis activity in experimental infections was isolated from *Streptomyces vinaceus* and called vinactane[469]. Subsequently it was proved to be identical to viomycin[469].

Chemistry

Viomycin is a strongly basic polypeptide, which forms essentially neutral, crystalline salts[467,468,479]. On acid hydrolysis[479], carbon dioxide, ammonia and urea are liberated together with the amino acids L-serine, L-α, β-diaminopropionic acid[479,480] and L-β,ε-diamino-n-caproic acid (also called L-β-lysine)[481–483]. In addition a guanidino compound, viomycidine, has been isolated[471,479,484]. Dyer, Hayes and Miller found the structure of viomycidine to be 3-guanido-1-pyrroline-2-carboxylic acid (*Figure 1.10a*) on the ground of its physical properties and degradation results[484]. This was confirmed by Bowie, Johnson and Thomas[485], who suggested at the same time that viomycidine is present in the antibiotic as a cyclized structure (*Figure 1.10b*),

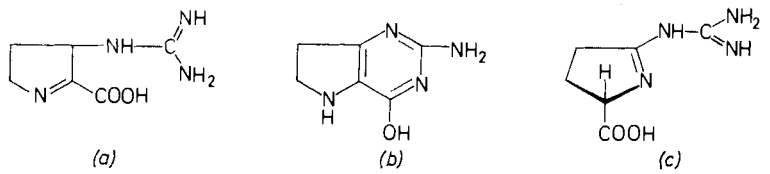


Figure 1.10. Proposed structure for viomycidine

a derivative of 6,7-dihydro-5*H*-pyrrolo[3,2-d]pyrimidine. This later structure for viomycidine is in disagreement with the structure[470] recently proposed as 2-guanido-$\triangle^1$-pyrroline-5-carboxylic acid (*Figure 1.10c*) with R configuration of the asymmetric centre. Partial hydrolysis of viomycin, separation and degradation of the resulting peptides led to a preliminary structure for viomycin (*Figure 1.11*)[471]. It seems that neither of the two

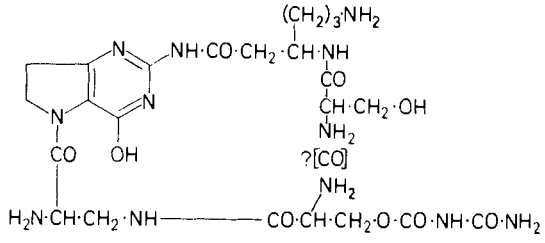


Figure 1.11. Proposed structure for viomycin[471]

serine amino groups are free in the antibiotic itself and it is possible that they might be linked through a carbonyl group.

This structure, however, has been challenged very recently by Dyer, Kellogg, Nassar and Streetman[486]. Because the structure in *Figure 1.11* does not satisfy some of the physical and chemical data obtained for viomycin, they proposed an alternative structure (*Figure 1.12*).

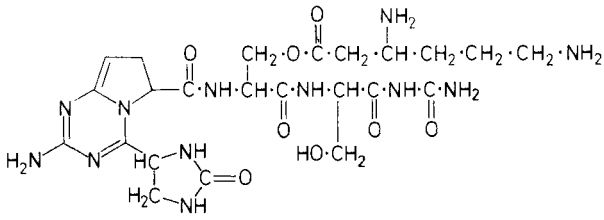


Figure 1.12. Alternative structure for viomycin[486]

The pyrrolo-[1,2-*a*]-*s*-triazine ring system, formed by viomycidine and α,β-diaminopropionic acid, explains the ultra-violet absorption of viomycin. This ring system may also be responsible for the antimicrobial activity of viomycin[486], because several dihydro-*s*-triazines possess a remarkable activity towards many micro-organisms. Also this formula cannot yet explain all the results reported in the literature and the final structure for viomycin is thus still undetermined.

Antimicrobial activity

The antimicrobial activity of viomycin is mainly restricted to *M. tuberculosis*. It is bacteriostatic for *M. tuberculosis var. hominis* H37Rv in concentrations of 2·5 to 12·5 μg/ml. Other gram-positive and gram-negative bacteria are inhibited only by larger amounts of the antibiotic[467,468,487] (*Table 1.7*), and against fungi, protozoa and viruses it is completely inactive. Resistance to viomycin appears on repeated subculture as quickly as does resistance to streptomycin, and it remains stable through many transfers[488,489]. Viomycin was shown to have a definite chemotherapeutic activity against experimental infections due to *K. pneumoniae*, *E. typhosa* and *Proteus vulgaris*, when administered orally or subcutaneously[487], yet its main characteristic was the marked suppressive effect on tuberculous infections in experimentally inoculated mice[487,490,491] and guinea-pigs[489,492,493]. It was, however, observed that the development of tuberculosis in guinea-pigs was only retarded and progressed again after the treatment with viomycin had been discontinued[492]. Two types of methods are described for determining product potencies, either a cylinder-plate method using *B. subtilis* ATCC 6633 as test organism, or a turbidimetric method using *K. pneumoniae* ATCC 10 031 as test organism. The viomycin standard is the anhydrous base, which has been assigned a potency of 1,000 μg/ml.[111]

Clinical use

Viomycin is used in combination with other tuberculostatic agents in the treatment of tuberculosis[494]. Because of its toxic side effects its use is not recommended for patients who respond to the classical forms of treatment.

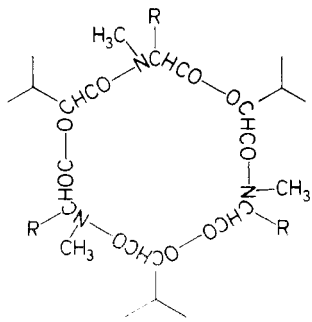
Table 1.14. Polypeptide antibiotics produced by fungi

Name	*Organism*	*Antibiotic spectrum*	*Mol. wt.*	*Amino acids**		*Other components*	*Ref.*
				D	L		
Antibiotic I.C.I. 13 599	*Paecilomyces sp.*	Antitrypanosome			Abut, β-Ala, β-Hyleu, γ-MePro		509
Avenacein	*Fusarium avanaceum*	*M. phlei*			Me-Val	D-α-hydroxy-isovaleric acid	502
Cephalosporins							
Cephalosporin C,N	*Cephalosporium sp.*	Gram-pos. bacteria,	~350	α-aminoadipic acid			
		Gram-neg. bacteria		Penicillamine			14
Enniatins							
Enniatin A, B, C	*Fusarium oxysporum*	Acid-fast bacteria	~650		Me-Val, Me-Ile, Me-Leu	D-α-hydroxy isovaleric acid	503, 510
Fructigenin	*Fusarium fructigenum*	*M. phlei*			Me-Val	D-α-hydroxy-isovaleric acid	502
Lateritiin (I, II)	*Fusarium lateritium*	*M. phlei*			Me-Val	D-α-hydroxy-isovaleric acid	502
Penicillins	*Penicillium sp.*	Gram-pos. bacteria	350	Penicillamine		{Phenylacetic acid, valeric acid	10–13
Sambucinin	*Fusarium sambucinium*	*M. phlei*			Me-Val	D-α-hydroxy-isovaleric acid	502

Viomycin, like the other polypeptide antibiotics, has certain nephrotoxic effects, which are manifested by albuminuria and cylindruria[142,495,496]. Blood electrolyte disturbances[495], allergic reactions, partial hearing loss and pain at the site of injection have been observed[496]. These effects, however, are mainly dependent on the dosage and are rare if the recommended doses are applied[497]. Intermittent dosage schedules and the application of viomycin-pantothenate[498–501] have been reported to reduce the toxic side effects (*Table 1.8*).

POLYPEPTIDE ANTIBIOTICS PRODUCED BY FUNGI

All the known peptide antibiotics (*Table 1.14*) produced by *Fusaria* were isolated in 1947. Cook, Cox, Farmer and Lacey[502] reported on five antimicrobial substances isolated from *F. lateritium*, *F. avanaceum*, *F. sambucinium* and *F. fructogenum* and called lateritiin I, lateritiin II, avenacein, sambucinin and fructigenin. These compounds are mainly active against *Mycobacterium phlei*. Plattner and Nager[503] characterized enniatin A, B and C isolated from *F. oxysporum*, which are mainly active against *Mycobacterium tuberculosis*, *M. phlei*, *B. subtilis*, *Staph. aureus* and *E. coli*. All these antibiotics belong to the depsipeptides, a large class of peptide-related compounds, which are built up of hydroxy and amino acid residues joined by amide and ester linkages. The structures originally proposed for the enniatins had to be revised after their total synthesis by Russian[504–506] and Swiss workers[507,508] (*Figure 1.13*). The structures of the other antibiotics are still unknown. None of these compounds is of clinical use.



Enniatin A R = Bu^s
Enniatin B R = Pr^i
Enniatin C R = Bu^i

Figure 1.13. Structures of enniatin A, B and C

Several reviews are available of the most important members of this group, the penicillins[10–13] and the cephalosporins[14], which from the point of view of biogenesis can be regarded as peptides. They are not, however, considered here, because they are not real polypeptide antibiotics.

POLYPEPTIDE ANTIBIOTICS ISOLATED FROM DIFFERENT SOURCES

Only a few peptide antibiotics have been reported, which are not produced by bacilli, streptomyces and fungi. These are compiled in *Table 1.15*. For

Table 1.15. Polypeptide antibiotics from different sources

Name	*Source*	*Antibiotic spectrum*	*Mol. wt.*	*Amino acids*	*Other components*	*References*
Coliformin	*E. coli-Aerobacter aerogenes* type	Fungi	~4,000	Ala, Asp, Glu, Gly, Leu, Lys, Ser, Val	Glucose, xylose	514
Comirin	*Bacterium antimyceticum*	Fungi		Ala, Arg, Asp, Glu, Gly, Ile, Leu, Lys, Ser, Tyr, Val, Thr, Dab	Unknown	515
Calf thymus peptide	Calf thymus	Antimycobacterial activity	4,300–10,000	Ala, Arg, Asp, Glu, Gly, His, Ile, Thr, Leu, Lys, Met, Phe, Pro, Ser, Tyr, Val		513
Diplococcin	*Streptococcus lactis*	Streptococcus cremoris, gram-pos. bacteria		Arg, unidentified amino acids		516
Nisins (A,B,C,D)	*Streptococcus lactis, cremoris*	Gram-pos. bacteria	~7,000	Ala, Asp, Gly, His, Ile, lanthionine, β-methyllanthionine, Leu, Lys, Met, Pro, Val		517 518
Serratamolide	*Serratia marcescens*	Gram-pos., gram-neg. yeast, fungi	514	L-Ser	D-β-hydroxydecanoic acid	511, 512
Viscosin	*Pseudomonas viscosa*	Antimycobacterial activity		Gly, Leu, Ser, Thr, Val		519–522

most of them only a qualitative amino acid analysis has been published although they have been known for several years. This may be due to their considerable molecular weights, which exceed practically all the molecular weights obtained for the other peptide antibiotics. Only the structure of serratamolide (*Figure 1.14*), a small depsipeptide, has been elucidated[511] and

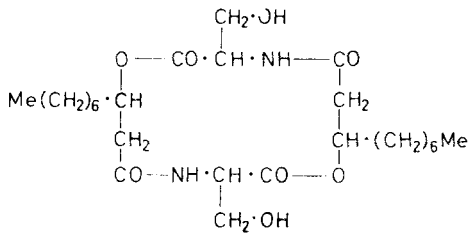


Figure 1.14. Structure of serratamolide

proved by synthesis[512]. A peculiar compound is the strongly basic polypeptide antibiotic isolated from calf thymus[513].

The only members of some practical importance are the nisins, which are used in the food industry as preservatives.

BIOSYNTHESIS OF POLYPEPTIDE ANTIBIOTICS

The intensive studies on the genetic code and on the proteins in recent years have led to a fairly good understanding of the mechanism of protein biosynthesis[523]. The biosynthetic mechanism involved in the formation of peptides has not yet been studied in equal detail. Some physiologically active peptides like bradykinin and angiotensin are known to be derived from proteins by a specific enzymatic hydrolysis. Other peptides, like glutathione[524,525], ophthalmic acid[525], the nucleotide-pentapeptide from *Staph. aureus*[526] and γ-polyglutamic acid[527] have been shown to require for their synthesis only a soluble enzyme system. Their biosynthetic mechanism is therefore entirely different from that of the proteins. Such a different type of mechanism has also been demonstrated lately to be involved in the synthesis of peptide antibiotics.

Mach, Reich and Tatum were able to demonstrate an inhibition of the biosynthesis of protein in cells of *B. brevis* by chloramphenicol and puromycin without affecting the synthesis of tyrocidine[528]. Several analogues of amino acids were found, which inhibited the biosynthesis of tyrocidine without affecting that of protein and vice versa. In contrast to protein synthesis, the production of tyrocidine did not depend on the continuous synthesis of RNA. Furthermore, environmental factors were able to control the relative amounts of the different tyrocidines synthesized by genetically homogeneous cultures[66]. Addition of phenylalanine to the culture medium resulted in the almost exclusive synthesis of tyrocidine A, whereas the unsupplemented culture produced tyrocidine A, B and C. In the presence of tryptophan, a new form of tyrocidine, called tyrocidine D, containing three tryptophan in place of three phenylalanine residues, was produced. This lack of absolute requirement for specific amino acids in the formation of a peptide bond is in contrast to the strict specificity of sequential incorporation of amino acids

into proteins. This influence of the relative concentrations of amino acids in the medium on the primary structure of the polypeptide chain can be, besides mutation, an explanation for the fact that many peptide antibiotics are groups of closely related substances.

In contrast to these results, the process of tyrocidine and gramicidin biosynthesis has been compared to protein synthesis, but this evidence was obtained in cell-free systems of *B. brevis*[529,530]. Yet a difference in the mechanism of protein and antibiotic synthesis has also been reported for gramicidin S[531–533], polymyxin B[534,535] and mycobacillin[536] and seems now to be fairly well established.

Bernlohr and Novelli[537] reported that, although the mechanism of antibiotic synthesis differs from protein synthesis, there appears to be a competition between the two processes for the amino acids available in the cell. During active cellular growth with high protein synthesis, practically no bacitracin was produced. In contrast, bacitracin synthesis was high, when the requirements for protein synthesis were low, as at the end of the log phase or when protein synthesis was inhibited. The production of bacitracin, and some other antibiotics, seems to be related in some way to the spore-forming metabolism[537]. In this phase a great part of the cell wall is dissolved, while protein production remains very low.

The origin of the D-amino acids has been traced in a few cases and it has been found that D-amino acid residues are more efficiently labelled when the corresponding radioactive L-amino acids are added to the growing culture than when the radioactive D-isomers are used. D-Amino acids have been found to be competitive inhibitors of peptide synthesis. These experiments suggest that free D-amino acids are not obligatory intermediates in the formation of peptide antibiotics[538–542]. The inversion of the configuration seems to occur at the peptide level[543].

Little is known concerning the mechanism of biogenesis of the often occurring *N*-methylamino acids. There is evidence that glycine and L-valine are the direct precursors of sarcosine[539,544,545] and *N*-methyl-L-valine[539] in actinomycin biosynthesis and that the methyl group is provided by methionine[545,546].

The chromophores of some of the peptide antibiotics may also be synthesized from amino acids. Thus the phenoxazine ring of actinomycin originates from tryptophan[546,547].

MECHANISM OF ACTION OF POLYPEPTIDE ANTIBIOTICS

Several reviews deal with the mechanism of antibiotic action[548,549]. With respect to their primary mode of action, the antibiotics have been divided into three main groups: (*a*) those affecting the structure, or the synthesis, or both of the bacterial cell wall; (*b*) those affecting the function, or the synthesis, or both of the protoplast membrane; (*c*) those affecting protein and nucleic acid synthesis. According to this classification, the tyrocidines, gramicidins and the polymyxins belong to group (*b*), whereas the bacitracins cannot yet be classified with certainty. The chemically completely different linear gramicidins do not belong in either group; they act as uncouplers of oxidative phosphorylation. Nothing is yet known about the mode of action of amphomycin and viomycin.

Tyrocidine was found to produce a rapid decrease of respiration of staphylococci[550]. Its action is directed towards the osmotic barriers resulting in a massive leakage of amino acids, purines, pyrimidines, phosphates and phosphate esters from the cell into the medium[551]. The resulting dilution of essential metabolites practically stops any biosynthetic process. This action is independent of growth.

An analogous effect is exerted by gramicidin S and by the polymyxins. The alteration in the permeability of the cell membrane was clearly demonstrated by the use of *N*-tolyl-α-naphthylamine-8-sulphonic acid, which gives rise to fluorescence when in contact with proteins. Little fluorescence was observed when intact cells were treated with this substance. Under the influence of polymyxin, however, the dye was able to penetrate into the cell where it reacted with the cytoplasmic proteins giving brilliant fluorescence[552]. Combination between the protoplast membrane of *B. megaterium* and polymyxin has been demonstrated by using a fluorescent derivative of polymyxin[553]. This fixation of polymyxin, which is antagonized by cations, seems to be due to a reaction with ionized phosphate groups in the membrane[554–556]. This evidence has been further substantiated by the observation that polymyxin-sensitive cells contain more lipid phosphorus than resistant cells, and that they also bind more of the antibiotic[557]. Electron microscope studies have revealed the disorganization of the cell surface occurring upon treatment with polymyxin[558,559] or colistin[560].

In their similar action on the cell membrane the tyrocidines, gramicidins and the polymyxins resemble other surface active agents. Like these, they contain also lipophilic and lipophobic groups, which, as has been revealed by studies on gramicidins, may be separated in the molecule by being fixed on different sides of the molecular plane.

Inactivation of certain enzyme systems involved in the oxidative metabolism of sensitive organisms by polymyxin[558,561] and colistin[562] has also been reported. This, however, might be a secondary effect[562]. Bacitracin has been reported to interfere with cell wall synthesis. It causes *Staphylococcus aureus* to lyse[563,564], to form protoplasts[565] and to accumulate cell wall precursors[564,566–568]. The incorporation of radioactive amino acids into cell wall mucopeptides is inhibited.[566] Bacitracin has further been found to prevent *Staphylococcus aureus* from synthesizing β-galactosidase[564,569], yet it does not interfere with the incorporation of radioactive lysine into cells[570]. In experiments with *Staphylococcus aureus*, bacitracin and penicillin were shown to share a common binding site on the membrane[571], a result which could not be confirmed in similar experiments with *B. megaterium*[572]. Recently a direct effect of bacitracin on the cytoplasmic membrane has been demonstrated, and it was suggested that the inhibition of cell wall synthesis could be a secondary effect[572].

The role of zinc ions in the enhancement of the antimicrobial activity of bacitracin is not yet clear. It has been reasoned that in the complete absence of Zn^{2+} bacitracin would possess no bacteriostatic activity[564].

An analogous mechanism in the mammalian cell may explain the high toxicity of these peptide antibiotics. Their specific nephrotoxicity may be due either to a difference of the structure between renal cell membranes and those in other cells or it may be due to the high concentration of the antibiotics in the renal system.

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2

NON-STEROIDAL ANTI-INFLAMMATORY DRUGS

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INTRODUCTION

The study of inflammation and anti-inflammatory drugs gained considerable impetus from the demonstration in 1949 of the antirheumatic action of cortisone. Despite a considerable amount of research and an even greater amount of speculation, the mechanism of action of anti-inflammatory compounds is still largely unknown. Indeed, the new anti-inflammatory drugs which have appeared during the past few years have been developed on the basis of empirical screening procedures. A fundamental difference in mode of action separates the glucocorticoids, such as cortisone, from chemically heterogeneous groups of compounds, including the salicylates and pyrazolones, which exhibit some similarities in their anti-inflammatory effects. Whilst this review excludes the former group from primary consideration, comparative data relating to corticosteroids are included where appropriate.

THE NATURE AND MECHANISM OF INFLAMMATION

Inflammation may be defined as a series of localized tissue responses to foreign stimuli, which may be biological (e.g. bacteria, fungi, viruses), chemical (e.g. croton oil) or physical (e.g. X-rays, ultra-violet light). Apart from external stimuli, it now appears than an endogenous factor, the autoimmune reaction, may initiate an inflammatory response.

It is customary to make a distinction between acute inflammation, which is the response of tissues to severe but transient stimuli (e.g. many bacteria or irritant chemicals), and chronic or granulomatous inflammation, which occurs when the stimulus is persistent and usually weak. Acute inflammation is a predominantly vascular process which is characterized by erythema (dilatation of arterioles, small veins and capillaries), stasis (reduced blood flow in the smaller vessels), increased capillary blood pressure, increased vascular permeability, leucocytic infiltration into the affected area, and pain. Whilst the detailed changes vary to some extent according to the particular species and organs involved and the nature of the inciting agent, there is, nevertheless, an underlying pattern of response which suggests that common intermediate factors may be responsible for the various phenomena of inflammation.

A considerable amount of research has been aimed at the identification of these intermediate factors. Several naturally occurring substances such as histamine, 5-hydroxytryptamine, bradykinin and certain plasma globulins have been shown to have appropriate pharmacological properties. To prove that such substances have a role in producing inflammatory changes is, however, difficult and the likelihood of their doing so must be decided by a careful assessment of the evidence. A detailed analysis of the mechanism of

inflammation is outside the scope of this review, and only those aspects which impinge on the methods of discovering new anti-inflammatory drugs and of studying their mode of action will be discussed. A basis for further study will be found in four recent symposia[1–4].

Chronic inflammation is essentially a reparative process in which various types of cell, other than the polymorphonuclear leucocyte, invade the area and in which connective tissue is laid down to replace necrosed parenchymal tissue.

Although it is now universally accepted that inflammation serves the useful purpose of minimizing the damaging effect of the causative irritant, there are circumstances when both acute and chronic inflammation appear to be wholly detrimental to the host, as, for example, in rheumatic and allergic diseases. It is the clinical problem posed by these diseases which has stimulated most of the research into the development of anti-inflammatory drugs. However, for the purpose of this review, an anti-inflammatory drug is defined as a compound which inhibits the whole or any portion of an acute or chronic inflammatory reaction, regardless of whether the drug is clinically useful.

Erythema

Erythema, the gross manifestation of dilatation of the smaller arterioles, is usually the earliest macroscopically visible sign of inflammation. It is of particular importance in the present context since tests involving measurement of erythema are among the most sensitive means of discovering anti-inflammatory activity. Most of the substances capable of increasing vascular permeability also produce vasodilatation; erythema, without associated exudation, can often be produced by low concentrations of such substances. However, the persistence of the erythema of inflammatory reactions contrasts with the transient nature of that produced by histamine or bradykinin.

Various methods have been described for producing erythema experimentally in animals and man, the most important being those involving the use of nicotinic esters or of ultra-violet light. It seems clear that the appearance, course and mechanism of erythema varies according to the method of inducing it. Striking cinephotomicrographic observations suggest that nicotinates act directly on the vessels[5,6]. No direct evidence for a mediating substance in nicotinate erythema has been forthcoming: certainly histamine does not seem to be involved[7]. The mechanism of ultra-violet erythema, which appears to be different from that of nicotinate erythema, has been reviewed by Blum[8]. The participation of mediating dilator substances is inferred from the fact that, although radiation is absorbed in the epidermis, dilatation occurs in vessels in the dermis. The evidence suggests that at least two dilator factors may be involved, and neither is histamine.

When nicotinic acid esters are applied to the skin of patients with rheumatoid arthritis, an anomalous vasoconstriction frequently occurs instead of the usual erythema and was at first thought to offer diagnostic possibilities[9]. It is now apparent, however, that the effect is attributable to the therapeutic use, by such patients, of acetylsalicylic acid, which is remarkably potent in retarding the development of the reaction[10,11].

Further study of the mechanism of erythema seems to be worthwhile in

view of the correlation between the anti-inflammatory activity of certain drugs and their suppressive effect on various types of erythema[11–13].

Increased Vascular Permeability

More research has been devoted to the study of increased vascular permeability than to any other aspect of inflammation, and several excellent reviews on the subject have recently appeared[14–16]. Increased vascular permeability here refers to the increased tendency for fluid and plasma proteins to pass through the vessel wall into the extravascular space. Various methods of studying the process have been described, usually involving either the assay of extravasated protein or protein-bound dyes such as trypan blue, or the measurement of the degree of swelling produced in a rat's paw after the subcutaneous injection of an irritant (see page 67).

Histamine and 5-hydroxytryptamine

The concept of an endogenous mediator of increased permeability was supported by the classical studies of Lewis[17] on histamine. The increase in vascular permeability is a biphasic process, the earlier transient stage, in some species at least, resulting from the release of histamine[16]. After suitable mild inflammatory stimuli, the tissue oedema may often be inhibited by antihistaminic drugs. In a limited sense, therefore, antihistaminic drugs may be regarded as having anti-inflammatory activity, but they are not discussed in detail in this review.

In the rat, which is relatively insensitive to histamine, and possibly also in the mouse, 5-hydroxytryptamine (serotonin) may be an important factor in the production of vascular permeability[18–20]. Thus, inflammatory oedema in these species may often be prevented by the administration of serotonin inhibitors such as bromolysergic acid diethylamide[21]. It is generally accepted, however, that histamine and 5-hydroxytryptamine cannot account for all the manifestations of vascular permeability in inflammation. Attention has been focused on possible mediators of higher molecular weight, especially peptides and proteins.

Leukotaxine

By fractionation of inflammatory exudates, Menkin found a substance, leukotaxine, thought to be a peptide, which increases vascular permeability and is also chemotactic. Menkin's studies of this and other inflammatory factors have been reviewed from time to time[22–24] and have been the target of criticism[25] and countercriticism[26]. Leukotaxine was acknowledged to be impure, and some workers have doubted whether the peptide which it contained was, in fact, responsible for the permeability-increasing activity of the original exudate. Subsequent work by Spector[27] demonstrated the presence, in inflammatory exudates, of peptides which increase vascular permeability; the amount of peptide remains constant during the course of formation of the exudate however, and does not reflect the development of the vascular permeability which is maximal at about 6 hours.

Whatever the nature and significance of leukotaxine, Menkin deserves credit for pioneering the chemical investigation of inflammation. Without adequate means of producing leukotaxine experimentally and of identifying

and assaying it, the study of anti-inflammatory drugs in terms of 'anti-leukotaxine' activity is not possible.

Kinins

A vasodilating peptide with smooth-muscle stimulating action was described by Rocha e Silva, Beraldo and Rosenfeld in 1949[28] and named bradykinin. It is produced by the action of trypsin or snake venoms on plasma. A similar substance, kallidin, was prepared shortly afterwards by Werle and Berek[29] using the protease kallikrein. Other peptides with similar properties have been described[30,31] and the general term 'kinins' or, when derived from plasma proteins, 'plasma kinins' has been recommended. The best-known kinin, bradykinin, has recently been shown to be a nonapeptide, and its structure confirmed by synthesis[32,33]. When injected subcutaneously into laboratory animals and man, bradykinin causes vasodilatation, increased vascular permeability, leucocyte infiltration and pain.

The properties of bradykinin render it, or some similar kinin, a plausible mediator for several, if not all, of the manifestations of acute inflammation[34]. The necessary substrates and proteases seem to be available[35,36] and it is quite likely that kinins are formed during inflammation but are usually too rapidly destroyed by local peptidases to be isolated. Additional evidence that kinins participate in inflammatory reactions has come from the finding by Muftie and Roch-Ramel[37] that the perfusate from a tuberculin reaction in the guinea-pig contains a kinin-like peptide. Moreover, Greaves and Shuster[38] found that injection of plasma kinin-forming substrates into a tuberculin-injected area of skin in normal subjects enhances the subsequent reaction. Plasma fractions devoid of substrate are inactive. Despite the qualifications of kinins to act as inflammatory mediators, it is surprising to find that anti-inflammatory drugs inhibit the activity of kinins only in a limited number of systems (see page 119).

Globulin permeability factor (Globulin P.F.)

It has been demonstrated[39–41] that a factor capable of increasing vascular permeability is formed when plasma of certain species is diluted with saline in contact with glass. The activity is associated with a globulin, the particular fraction varying from species to species. An inhibitor also occurs in plasma. Activation by dilution is attributed to the splitting of a P.F.-precursor/P.F.-inhibitor complex. As it is inhibited by the various known trypsin inhibitors and by dyflos (D.F.P., isoflurophate), Miles considered that globulin P.F. may be a protease, but no substrate has been demonstrated. Globulin P.F. may, in fact, be identical with kallikrein.

Spector[27] showed that after the intrapleural injection of turpentine in rats the increase in vascular permeability coincides with the development of permeability-increasing activity by the globulin fraction of the exudate. As the permeability of the vessels returns to normal so the globulin fraction loses its activity owing, apparently, to the appearance of an inhibitor. Further work is needed to clarify the properties and role of the permeability globulins in inflammation. Salicylate is reported to inhibit the activation of these globulins, and the effect of other known anti-inflammatory drugs in this respect would be of interest.

Leucocytic Infiltration

The appearance of large numbers of leucocytes at the site of injury is a characteristic feature of inflammation. In the earliest stages polymorphonuclear leucocytes predominate, but in time these give place to lymphocytes and other types of mononuclear leucocytes, some originating in the blood and others arising from the tissues. The microscopically-observed sequence of events giving rise to these cellular infiltrations has been thoroughly investigated and the passage of leucocytes through the vessel wall observed with the aid of the electron microscope[42,43]. By contrast, much less is known about the mechanism of the phenomenon.

The earlier literature has been well reviewed by Harris[25,44]. More recently, Hurley and Spector[45] have described a non-dialysable, heat-labile factor in serum and tissues which, unlike many extracts, is capable of causing immediate leucocytic migration when injected. Furthermore, Spector and Willoughby[46] have shown that a factor isolated from guinea-pig lymph-node cells, and another present in serum extract of rat liver, causes greater leucocytic migration than does any other permeability factor tested. It therefore appears that the factor(s) responsible for leucocytic migration are distinct from those which increase vascular permeability. Until these factors have been better characterized, and until simpler techniques can be evolved, the effects of anti-inflammatory drugs on this aspect of inflammation cannot easily be studied. Despite these difficulties, Saxena[47] was able to show a significant effect of several anti-inflammatory, vasoconstrictor and central nervous system depressant drugs on the leucocyte content of inflammatory exudate, formed in plastic sponge implanted subcutaneously in rats. Phenylbutazone, sodium aurothiomalate, corticotrophin and hydrocortisone all reduced the cell count, without affecting the weight of the exudate. The effect of anti-inflammatory and other compounds on the accumulation of leucocytes induced by intraperitoneal injection of a small volume of 0·9 per cent sodium chloride solution containing 0·2 per cent acetic acid was studied by Northover[48]. The mean polymorphonuclear leucocyte count in the fluid was significantly reduced by a number of corticosteroids but by none of the non-steroidal anti-inflammatory agents tested, including sodium salicylate and sodium phenylbutazone.

Pain

Pain is not only one of the cardinal characteristics of inflammation, but the most significant one in clinical inflammatory disease; it is often the principal target of anti-inflammatory treatment. However, pain is often of non-inflammatory origin and may be relieved, regardless of its origin, by agents acting in several different ways.

Little is known about the mechanism of inflammatory pain. Kelly[49] has discussed the question of pressure on nerves. The accumulation of exudate in an abscess leads to pain which is immediately relieved when the abscess is incised. On the other hand, greater tension exists in tissues affected by angioneurotic oedema although no pain is produced. It is known that externally-applied pressure, insufficient to elicit pain in normal tissue, causes pain in an inflamed area. Randall and Selitto[50] used this hyperalgesic state as the basis for their now widely used test for analgesic drugs.

By analogy with other manifestations of inflammation, it is reasonable to consider the possibility that the hyperalgesia also results from the formation of a chemical mediator such as an amine or a kinin. Histamine, acetylcholine, 5-hydroxytryptamine and bradykinin all produce pain when injected subcutaneously or intradermally in man, or when applied to the exposed base of a blister[51,52]. Bradykinin is of particular interest since it appears to be identical with the pain-producing substance described[52] in 1957 and is far more potent than the other algesic agents. For example, Cormia and Dougherty[53] found that bradykinin produced pain when injected intradermally in concentrations of from 10^{-6} to 10^{-8} whereas histamine is not active below 10^{-5}. Guzman, Braun and Lim[54] studied the vocal response of dogs and cats to various algesic agents and provided evidence that this is an indicator of pain-production in the animals. They found bradykinin to be the most potent agent tested, having at least 50 times the activity of histamine and 5-hydroxytryptamine.

Gilfoil and Klavins[55] produced oedema and hyperalgesia in the rat-paw by the local injection of various agents, including trypsin, brewers' yeast and dextran. However, oedema of similar magnitude produced by injecting histamine, 5-hydroxytryptamine or bradykinin was not associated with hyperalgesia. In yeast-induced inflammation, acetylsalicylic acid relieves the hyperalgesia but does not affect the oedema, suggesting that the two phenomena have different causes. Further work[56] demonstrated the presence of bradykinin in perfusates of yeast-injected paws but it is also present after acetylsalicylic acid treatment. A heat-labile substance, inactivated by dyflos, is also demonstrable. Acetylsalicylic acid does not modify the hyperalgesia produced by endogenous 5-hydroxytryptamine resulting from the injection of turpentine or dextran.

In view of the continuous nature of the pain and hyperalgesia in inflammation, it follows that, if bradykinin is responsible, it has to be continuously released or its usually rapid destruction by tissue kininase has to be inhibited in inflamed tissue.

Catecholamines and Inflammation

Spector and Willoughby[57,58] have pointed out that the vascular changes in the acute inflammatory reaction may be due to the destruction of local vasoconstrictor substances such as adrenaline. Evidence in favour of this mechanism includes the observation that increased capillary permeability after thermal injury is suppressed by iproniazid and other monoamine oxidase inhibitors. Such inhibitors are known to inhibit the conversion of adrenaline, noradrenaline, 5-hydroxytryptamine and other amines to inactive metabolites. The authors provide evidence that the action of the monoamine oxidase inhibitors on capillary permeability is dependent on their anti-enzymic activity and not on some other unrelated property. Nevertheless, the evidence remains indirect; an attempt to detect pressor amines in the plasma of burned animals was unsuccessful. The potentiating effect of bretylium and the antagonistic action of an adrenolytic substance, dibenamine, on the action of iproniazid suggest that it is local depots of adrenaline rather than noradrenaline or 5-hydroxytryptamine which are involved. Independent support for this suggested role of catecholamines

comes from studies[59] which demonstrated the presence, in human skin, of chromaffin cells closely associated with the small venules and arterioles. The evidence suggests that such chromaffin cells contain an adrenaline-like substance and that various types of inflammatory stimuli cause the cells to become degranulated.

Recently, Willoughby and Spector[60] have broadened the hypothesis by showing that the vascular response to injury depends also on the inactivation of noradrenaline, DOPA and dopamine, and that inhibitors of DOPA decarboxylase or dopamine β-oxidase reduce vascular permeability in thermal burns in the rat.

Chronic Inflammation

Chronic inflammation is a process which gradually replaces the acute condition when the irritant is either a mild and unreactive substance (e.g. silica, metals and carrageenan) or certain micro-organisms (e.g. *Mycobacterium tuberculosis* or *M. leprae*). It is characterized by a cellular infiltration which is more heterogeneous than that of acute inflammation, polymorphonuclear leucocytes being replaced by monocytes, macrophages, histiocytes and fibroblasts. As the duration of the chronic process is longer, an extensive area of tissue necrosis results, leading to the repair process of granulation tissue formation. This consists of the deposition of connective tissue comprising increasing quantities of the fibrous protein, collagen, in a mucoprotein-containing matrix. Collagen is produced by fibroblasts, the initial synthesis occurring intracellularly and the molecule enlarging by extracellular polymerization. The mucopolysaccharides are also derived from connective tissue cells. In the complex process of chronic inflammation there are several points at which anti-inflammatory compounds may intervene. Many investigators have studied the effects of anti-inflammatory drugs on the weight of granulation tissue produced under standard conditions; others have sought to establish the particular process in which the drug intervenes.

The commonest forms of chronic inflammation occurring in man are rheumatoid arthritis and tuberculosis, the pathology of which has been the subject of intensive study. For laboratory purposes, however, there are obvious advantages in having a non-viable stimulus which gives a reproducible inflammation. Numerous techniques have been evolved, including the subcutaneous or intraperitoneal injection of silica or carrageenan, or the subcutaneous implantation of cotton-wool pellets or polyvinyl alcohol sponge[61,62].

TESTS FOR ANTI-INFLAMMATORY ACTIVITY

The number of tests that have been devised for measuring the anti-inflammatory activity of compounds is legion. Those described below have been chosen largely for their ability to show diverse types of anti-inflammatory activity, preference being given to those which are in common use and are therefore referred to most often in the section on anti-inflammatory compounds later in this review. In addition, a number of techniques that may not be widely used, but which show certain important facets of inflammation, have been included.

The methods used to provoke inflammation include hypersensitivity reactions, physical procedures, e.g. ultra-violet light and heat, foreign chemicals of inorganic or organic nature, and the biochemical inflammatory mediators themselves, e.g. 5-hydroxytryptamine, histamine, bradykinin. The intensity and nature of the inflammatory reaction depends not only on the type of inflammatory mediator that is injected or released *in situ*, but also on the species in which the reaction occurs. Thus Sparrow and Wilhelm[63] have shown that the permeability responses of the skin capillaries to histamine, 5-hydroxytryptamine and compound 48/80 vary widely and independently in the rat, guinea-pig and rabbit. Histamine is highly, and about equally, potent in guinea-pigs and rabbits, but only one twentyfifth as potent in rats. 5-Hydroxytryptamine is 11 times more potent than histamine in rats, but has negligible potency in guinea-pigs and rabbits.

Spector and Willoughby[64], in reviewing techniques for anti-inflammatory testing, emphasize that inflammation is a series of physiological events occurring in orderly sequence, that no drug is equally effective in suppressing all aspects of it, and that some act on only one particular component of the reaction.

The methods described below for determining anti-inflammatory activity have been divided into three main groups, based on the nature of the physiological response evoked by the traumatic agent. These are (*a*) erythema, (*b*) increased vascular permeability and oedema, and (*c*) formation of granulation tissue. In addition, there are tests based on immunological responses in which one or more of the above components may be involved. In these tests it is possible that drugs may affect the inflammation or the immunological systems concerned.

A final group of miscellaneous tests is also included, but no attempt has been made to differentiate these on the basis of the physiological responses.

Erythema

Ultra-violet erythema in the guinea-pig

The use of this technique for the screening of anti-inflammatory compounds, first described by Wilhelmi[65], has since been modified by other workers[66–68]. The method described by Adams and Cobb[68] is as follows. Shaved albino guinea-pigs are dosed orally with the drug 30 minutes before an exposure of 20 seconds to ultra-violet light. Two hours later the degree of erythema is estimated visually on a scale 0 to 4 by an observer who is unaware of the dosage schedules.

Winder, Wax, Burr, Been and Rosiere[66] showed that, at reasonable dose-levels, there is a good correlation between the erythema-delaying action and antirheumatic action of non-steroidal compounds. Of over 100 pharmacologically active agents examined, only 10 pyrazole, salicylate and cinchophen derivatives delay ultra-violet erythema; anti-inflammatory glucocorticoids are inactive.

The doses of antirheumatic drugs needed to reduce erythema produced by the application of thurfyl nicotinate to the skin of the guinea-pig agrees well with those required to inhibit ultra-violet erythema[12].

Increased Vascular Permeability and Oedema

Rat-foot oedema

This type of test is the one most often used in the investigation of anti-inflammatory compounds. It is an acute experiment in which a noxious agent is injected into the subplantar region of the rat's paw. The characteristic response is an oedematous swelling of the foot, which appears within minutes of injection and which seems to be an almost purely vascular reponse. Microscopically it has been shown that the small vessel bed is dilated early in the reaction and remains so for a number of hours through the peak of the response[69]. If Evans's blue is injected intravenously before the hyperaemia commences, the foot becomes blue owing to the passage of plasma albumin, with which the dye is associated, into the affected area. A similar oedema of the feet is produced by the intraperitoneal or intravenous injection of egg-white or dextran (anaphylactoid reaction) but in these circumstances there is also a generalized oedema of the snout, ears and tail region[21,70–72].

Some of the substances used to produce oedema of the rat-paw are listed in *Table 2.1.* The general technique for this method is as follows. The inflammatory agent is injected into the subplantar region of one of the hind

Table 2.1. Substances used to produce oedema

Inflammatory mediators	*References*	*Substances releasing inflammatory mediators*	*References*
5-Hydroxytryptamine	18, 73–81	Dextran	18, 55, 76, 79–81, 85–88
Histamine	18, 76	Egg-white or ovomucoid	18, 76, 81, 89
Bradykinin	82–84	Carrageenan	81, 90–92
		Formalin	77, 79, 80, 81, 85, 86, 93–96
		Brewer's yeast	55, 81, 91, 97–101
		Silver nitrate	102
		Turpentine	86
		Trypsin	55
		Kaolin	103
		Mustard	81, 104
		Compound 48/80	18, 76, 77
		Potassium cyanide	85
		Monoiodoacetic acid	85
		Sodium fluoride	85
		Hyaluronidase	21, 77, 87

paws, and saline solution into the other; the anti-inflammatory drugs are administered before or at the same time. The degree of oedema is measured over the next few hours, and compared with undosed controls.

In assessing the potential anti-inflammatory activities of drugs against this type of reaction it must be remembered that a number of non-specific effects reduce the oedema[105]. Thus Lorenz[103], and Büch and Wagner-Jauregg[106] showed that hydrogen peroxide, sodium hydroxide, kaolin and talc have a nonspecific anti-inflammatory effect when administered intraperitoneally. These substances suppress oedema produced by egg-white, dextran, 5-hydroxytryptamine and formalin, probably as the result of an obscure reflex mechanism caused by irritation of the tissues at the site of

injection. Lorenz[103] suggests that, since phenylbutazone and salicylic acid cause irritation on injection, they and other anti-inflammatory agents should always be given orally. In this connection it is interesting to note that some flavine derivatives[107] produce a severe oedema when injected into the rat-paw[106]. This 'flavine' oedema is suppressed by a prior intraperitoneal injection of the same derivative.

The effects of various non-specific treatments on a variety of rat-foot oedemas have recently been comprehensively studied and a summary of these results is given in *Table 2.2*[108]. This shows that rapidly developing

Table 2.2. Influence of various factors on the intensity of local oedema induced by various inflammatory agents

	Carrageenan	*Yeast*	*Kaolin*	*Dextran*	*Compound* 48/80	5-*Hydroxy-tryptamine*
Room temperature and humidity	−	−	−	−	+	−
Hypothermia	−	−	±	±	−	+
Diuresis	−	−	−	+	−	−
Hypotension	−	+	+	+	+	−
Vasoconstriction	±	+	+	+	+	+
Adrenalectomy	−	−	−	−	−	−
ACTH or hydro-cortisone	+	+	−	+	+	+
Irritation	+	+	+	+	+	+

(*From* Garattini and Dukes[108], *by courtesy of* Excerpta Medica Foundation)
− no effect, ± weak inhibition, + strong inhibition

oedemas such as that due to dextran, are more susceptible to non-specific agents than are slowly developing ones typified by carrageenan oedema.

Winter[109] examined the effects of five antirheumatic and two anti-allergic compounds on rat-foot oedema produced by seven different inflammatory agents, and concluded that only carrageenan differentiates between the two classes of compound. He suggests that formalin, egg-white and 5-hydroxy-tryptamine are unsuitable for testing antirheumatic drugs. Other workers have concluded that carrageenan oedema is suitable for investigating anti-inflammatory drugs since it is little affected by drugs with other pharmacological properties[110,111].

The effect of the general toxicity of compounds on the development of oedema seems to have been completely overlooked by many workers. Winter, Risley and Nuss[112] rightly emphasize that doses of drugs that have been used to demonstrate inhibition of oedema have often been within the toxic range. Domenjoz[113] notes that the doses of phenylbutazone and 1,2-diphenyl-4-(phenylmercaptoethyl)pyrazolidine-3,5-dione (G 25671), which suppress formalin oedema by 50 per cent, are lethal in 23 per cent of the animals. The implications of these findings must be borne in mind when interpreting the

anti-inflammatory activities of compounds reviewed later, where details of dosage and route of administration are given so that the possible intervention of toxic factors can be assessed.

The measurement of oedema of the rat-paw—Many methods have been used to measure the degree of oedema; they include the increase in the weight of the paw[70], the increase in tissue-water[18], the measurement of silhouette area obtained photographically[114], the direct measurement of paw diameter[115] and the visual assessment of oedema and blueing[21]. Plethysmographic methods are superior to any of these techniques[90,91,116,117], and permit a rapid and reproducible quantitative assessment of swelling.

Mouse-foot oedema—A number of workers have used the mouse instead of the rat, producing oedema with a wide variety of agents[118–121].

Chemically-induced 'arthritis'

It is important to emphasize the difference between the previously mentioned acute oedema tests in the rat-foot, and chronic chemically-induced 'arthritis', an example of which is 'formalin arthritis'. In this type of experiment, first described by Selye[122], two or more injections of formalin are made into the subplantar area of the foot. After the acute oedema has subsided, the surrounding skin remains hyperaemic and after a few days the peri-articular connective tissue begins to proliferate, especially in the region of the ankle joint. Other irritants, such as mustard[67,123] and kaolin[124], have also been used to produce such chronic articular damage.

Oedema produced by thermal injury in the rat

Thermal injury is produced in the rat by using a standard burn of 55°C for 27 seconds. This is achieved by placing the shaved abdomen of a rat in contact with the end of a closed hollow brass cylinder, through which thermostatically controlled water is circulated. Increased capillary permeability in the injured area is measured by estimating the water content of skin and subcutaneous tissue or by a visual estimation of the leakage of circulating protein-bound trypan blue into the skin of the burnt area[125]. The water content is determined by excising the damaged tissue and weighing it immediately and then again after drying to constant weight.

Turpentine pleurisy in the rat

Experimental pleurisy is produced in rats by injecting 0·1 ml. of turpentine into the right pleural space. After a convenient interval, the resulting exudate is withdrawn from each pleural space and the volume measured[27].

Formation of Granulation Tissue

Granuloma pellet

Most of the techniques in current use are derived from the original method described by Meier, Schuler and Desaulles in 1950[126]. When pellets of cotton-wool, cellulose sponge or similar material are implanted subcutaneously into the abdomen of the rat and left for 7 days, granulation tissue forms around and into the pellets. After 7 days the animals are killed and the pellets, together with the granulation tissue, are removed and dried to

constant weight. The weight of granulation tissue may thus be readily calculated. Many modifications of this basic technique, which has been used in the main for anti-inflammatory assays of glucocorticoids, have been developed[104,112,127,128]. When corticosteroids and some of the more potent non-steroidal anti-inflammatory antirheumatic agents[112,127] are administered during the period of implantation, the weight of granulation tissue is reduced. The weaker non-steroidal antirheumatic agents, such as acetylsalicylic acid and sodium salicylate, are inactive in non-toxic doses under these conditions.

A valid criticism of the technique (similar to that mentioned by Lorenz[103] for rat-paw oedema) has recently been made by Cygielman and Robson[129], who observed that anti-inflammatory activity, determined by the cotton pellet test, of a group of substances derived from liquorice seems to be associated with irritant effects at the site of injection. They proceeded to show that the injection of known irritants such as antimony potassium tartrate and croton oil produces a significant reduction in granulation tissue. Robinson and Robson[130] suggested, in the light of further experiments, that the reason for this is that an anti-inflammatory factor is produced at the site of implantation of the irritant substance.

A somewhat different granuloma pellet technique has been described in which a piece of non-resorbable plastic sponge is inserted subcutaneously into rats, and the exudate and leucocytic infiltration into it are measured over the next 5 hours[47,131].

Granuloma pouch

This technique was first devised by Selye[132–134] and modified by Robert and Nezamis[135]. It has been used extensively for testing anti-inflammatory corticosteroids, but may also be used for assaying the more potent non-steroidal anti-inflammatory antirheumatic compounds.

A subcutaneous dorsal pouch is produced in the rat by the injection of 25 ml. of air; into this space is injected 0·5–1 ml. of 1 per cent croton oil in cotton-seed or similar oil. Over the following days a haemorrhagic exudate accumulates in the pouch, the wall of which becomes thickened and granulomatous. To increase the exudation of fluid into the cavity the air may be withdrawn 48 hours after the formation of the pouch. The animals are killed 7–14 days after the beginning of the experiment, the exudate drained from the pouch, and the volume measured. If desired, the pouch can be dissected and weighed.

It must be emphasized that the haemorrhagic exudate, which is mainly derived from the destruction of tissue cells, bears little relationship to the oedema produced in the acute tests, which is a result of increased vascular permeability[64]. The present test can therefore be assessed on either the volume of exudate or the weight of the pouch.

Hypersensitivity Reactions

Tuberculin reaction in the BCG-infected guinea-pig

Since there is a close relationship between connective tissue disorders and auto-immune diseases on the one hand, and delayed hypersensitivity on the other, it has been suggested that delayed hypersensitivity reactions offer

advantages as a test for screening antirheumatic drugs[136]. Such a test was used by Long and Martin for investigating the effects of a number of compounds[137].

The technique described by Floersheim[136] is as follows. Guinea-pigs are sensitized by the subcutaneous administration of BCG vaccine in Freund's complete adjuvant. An intradermal injection of tuberculin 4–8 weeks later, elicits a typical erythematous reaction which is evaluated by measuring the degree of infiltration, the area of erythema and its intensity 8 and 24 hours after the tuberculin injection. Drugs under test are given 30 minutes before and 8 hours after the tuberculin.

Anaphylactic 'arthritis'

This intra-articular immunological response has been produced in guinea-pigs by two slightly differing techniques[138,139]. The reaction can be produced by the intra-articular injection of egg albumin in guinea-pigs passively sensitized to this antigen. The course of the reaction is followed by measuring the transverse diameter of the joints over a period of 5 hours after the challenging injection. The swelling is quantitatively related to the amount of antibody nitrogen administered.

Adjuvant arthritis in the rat

In contradistinction to the various types of chemically-induced and anaphylactic 'arthritis' which have been mentioned above, adjuvant arthritis appears to simulate closely the inflammatory reactions seen in rheumatoid arthritis in humans. Stoerk, Bielinski and Budzilovitch[140] first reported the development of a chronic polyarthritis in rats, and this work was later elaborated by a number of other workers[141–144]. The reaction seems to be a type of delayed hypersensitivity response[145,146], although recent work suggests that this may not be so[147].

Several techniques have been used to produce the reaction, but basically the arthritic syndrome can be elicited by injecting killed mycobacteria in mineral oil into the plantar surface of the foot or intradermally into the tail. From the tenth day onwards the joints of one or all of the feet gradually become inflamed and are painful, particularly when pressure is applied; other inflammatory lesions occur in the ears, tail and lungs. These reactions are present in a severe form up to about the thirtieth day, after which they begin to subside[148]. The degree of inflammation can be assessed visually or by measuring the swelling with a micrometer.

Steroidal and some non-steroidal antirheumatic agents are capable of suppressing or reducing these inflammatory reactions[148].

Miscellaneous Techniques

A large number of other interesting and informative techniques have been devised and it is possible to mention only a few.

Methods have been developed[149,150] in which the permeability of the synovial membrane in the rabbit has been investigated, but some doubts as to the significance of these tests have been expressed[151,152]. Ungar, Kobrin and Sezesny[153] describe a technique for measuring the degree of inflammation produced in rats and guinea-pigs by the injection of various agents into

the skin. It is based on the weight gain of inflamed skin caused by the passage of plasma into this tissue. An increase of capillary permeability with subsequent oedema can be produced by applying croton oil to the ear of the rabbit[96] or mouse[67,154]. Inflammation has been produced in rats' skin by the local application of chloroform[67,155], and in rabbits' eyes by the application of mustard oil[96] or croton oil[156].

Whittle[157] has ingeniously extended the use of the mouse 'writhing' technique for the testing of analgesics, by measuring the increased capillary permeability in the peritoneal cavity produced by the writhing agent. In this test the intraperitoneal injection of acetic acid causes 'squirming' or 'writhing', and also increases the leakage of plasma-bound dye (Pontamine Sky Blue) into the peritoneal cavity. Non-narcotic analgesics inhibit both the squirming and the leakage of dye, and it is interesting to note that those non-narcotic analgesics which are most effective in inhibiting the leakage are also those which possess marked anti-inflammatory properties[158].

The development in mice of lung inflammation produced by the inhalation of aerosols of bacterial endotoxin and its reversal by anti-inflammatory agents is the basis of a recently developed assay[159]. An interesting and novel *in vitro* screening technique was developed by Mizushima, on the basis of the hypothesis that the mode of action of antirheumatic drugs is related to their interaction with proteins[160,161]. The effect of a variety of drugs on the heat stability of plasma proteins (fractions IV and V) was studied. Non-steroidal antirheumatic drugs have a strong stabilizing action on albumin (fraction V) but the non-antirheumatic analgesic–antipyretic drugs have little or no influence. It is claimed that some compounds selected by this technique are potent anti-inflammatory agents.

ANTI-INFLAMMATORY COMPOUNDS

Because of limited space and inadequate biological data, not all the compounds or chemical series for which anti-inflammatory activity is claimed in patent specifications are discussed in this review.

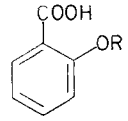
Hydroxybenzoates and Related Compounds

The history of the introduction of salicylates into medicine and details of the pharmacology and metabolism of these compounds have been presented in a number of reviews[162–170]. The scope and nature of much recent work in this field can be assessed from papers presented at the International Symposium on Salicylates held in London in 1962[171].

Salicylic and acetylsalicylic acids

The pharmacological and therapeutic relationship between acetylsalicylic acid (aspirin, *I*, R = COMe) and salicylic acid (*I*, R = H) is still not completely established. Experimentally, the anti-inflammatory activity of salicylic acid has been well established by many different tests[95,96,120,150,153,156,172–174]. When compared directly with salicylic acid, acetylsalicylic acid has equal or slightly greater activity[12,66,68,83,113,118,148,157,175–177] but there is little evidence that either compound is effective in inhibiting granuloma-type reactions[104,109,113,178,179].

The therapeutic activities of acetylsalicylic acid and sodium salicylate do not appear to have been compared directly in controlled trials, although it is generally considered that the former is superior[180–182]. However, Wood[183], on the basis of relief of stiffness, believes that their anti-inflammatory effects are equal. Wood has also pointed out the difficulties of deter-



(*I*)

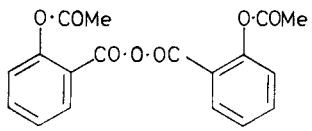
mining to what extent the therapeutic effectiveness of acetylsalicylic acid is due either to its analgesic or anti-inflammatory activity. The relative activities of the two compounds may depend on whether the acetylsalicylate ion possesses properties distinct from those of salicylate or whether it is simply more potent.

Acetylsalicylic acid is absorbed largely as such and although it is hydrolysed by blood and tissue esterases, appreciable amounts persist in the blood for up to 2 hours[163]. Lester, Lolli and Greenberg[184] consider that the period after ingestion, during which time it is present in the blood, corresponds to the duration of its analgesic action. Martin[163] suggests that acetylsalicylic acid may provide the optimal concentration of salicylic acid at the desired site over a suitable period of time, thus producing a superior clinical effect. Adams and Cobb[11] compared the inhibitory effects of oral doses of acetylsalicylic acid and sodium salicylate on erythema produced by thurfyl nicotinate in human volunteers. Acetylsalicylic acid was effective in doses of 225 mg, but sodium salicylate showed no activity in doses of 650 mg. These experiments indicate that the acetyl derivative may have a pharmacological effect in its own right without having to be hydrolysed to salicylate.

Despite the fact that acetylsalicylic acid has been used for many years, it is only recently that controlled trials have demonstrated its efficacy in the symptomatic treatment of rheumatoid arthritis[185–187]. The results of a 3 year multi-centre trial comparing cortisone acetate and acetylsalicylic acid, given in the lowest dosage needed to keep each patient symptom-free, indicate that the efficacy of both drugs is similar in almost all respects[187]. The condition of the patients after 3 years was better than at the start of the trial, but radiological deterioration occurred in both groups.

Derivatives of salicylic and acetylsalicylic acids

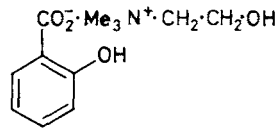
A number of derivatives, including salts, have been prepared with the aims of improving palatability and gastric tolerance. Aspirin anhydride (*II*) has been known for many years and has recently received renewed attention[188–190]. It was suggested that it gave higher acetylsalicylate blood levels



(*II*)

with less gastric disturbance. However, in a limited clinical trial, this compound was therapeutically less effective than acetylsalicylic acid and produced as much gastro-intestinal bleeding and more dyspepsia[191]. Levy and Gagliardi[192] showed that its gastro-intestinal absorption by humans is slow and incomplete.

A number of aluminium aspirin preparations have also been described, to which different structures have been assigned[193]. One of these compounds, aluminium aspirin NF, was found to be poorly absorbed compared with acetylsalicylic acid[194]. Aloxiprin (Palaprin) is a different form of aluminium aspirin, being a polymeric condensation product of aluminium oxide and acetylsalicylic acid, with the formula $Al_3O_2(C_6H_4(o\text{-}OCMe)\text{-}COO)_5$[193,195]. This compound is therapeutically as effective as acetylsalicylic acid; it produces less gastric irritation[196] and gastro-intestinal blood loss, but has a slight tendency to cause constipation[191].



(*III*)

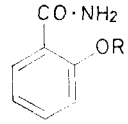
Choline salicylate (Arthropan, *III*) is claimed to have some pharmaceutical and therapeutic advantages over acetylsalicylic acid[197–203].

3- and 4-hydroxybenzoic acids

These compounds, in contradistinction to salicylic acid, are inactive in anti-inflammatory tests[68,172,175], and the little clinical evidence available indicates they are inactive in rheumatic fever[180,204].

Salicylamide

While there is reason to suppose that salicylamide (*IV*, R = H) and its derivatives have experimental analgesic and antipyretic properties[206,207], there is little evidence that it is an effective anti-inflammatory drug in



(*IV*)

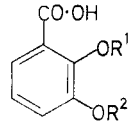
animals[66,68,208]. Unlike acetylsalicylic acid it does not inhibit bradykinin-induced bronchoconstriction in the guinea-pig[209]. Clinical trials suggest that salicylamide[210,211] and acetylsalicylamide (*IV*, R = COMe)[212] may be of value in various rheumatic conditions, but there is no indication that this drug is a clinically effective antirheumatic. A derivative, salicylamide-2-ethoxyethylether (ethosalamide, *IV*, R = $(CH_2)_2 \cdot Et$) was found in rheumatoid arthritis in a double-blind study to be inferior to acetylsalicylic acid[213].

Gorini, Valcavi and Zonta-Bolego, using formalin oedema in the rat, have

investigated the analgesic and anti-inflammatory activities of a large number of derivatives of salicylamide and cresotinamide given intraperitoneally[214]. Some of the compounds tested are given in *Table 2.3*. These workers found that, contrary to earlier evidence, salicylamide has analgesic and anti-inflammatory activity. Both types of activity were modified by substitution in the hydroxyl group. The introduction of an acetylamino group at C5 of *o*-allyloxysalicylamide to give compound 7 does not enhance the activity of the former compound 3, but compound 8, without the amide grouping, shows high activity. Since cresotinamide (compound 9) has anti-inflammatory and analgesic activities superior to those of salicylamide, the effect of substitution in the hydroxyl radical was examined. The allyl ether (compound 10) possesses anti-inflammatory and extremely high analgesic activities, and has shown clinical promise. However, whilst the introduction of a phenyl group (compound 12) reduces both activities, the introduction of a methyl group (compound 13) reduces only the analgesic activity. Ring substitution of cresotinamide at C5 does not increase activity in compound 15, and produces a compound having only anti-inflammatory properties in compound 16.

Dihydroxybenzoic acids

On the assumption that 2,3-dihydroxybenzoic acid (pyrocatechoic acid, *V*, $R^1 = R^2 = H$) is formed in humans after salicylate ingestion and that it may be the antirheumatic agent, clinical studies were carried out with this compound in rheumatic fever in which it appears to be superior to sodium salicylate although of lower toxicity, but ineffective in rheumatoid arthritis[215,216]. Other workers found the drug to be twice as active as salicylate in rheumatic fever and less toxic in therapeutic doses[204]. Trials with diacetylpyrocatechol-3-carboxylic acid (Movirene, *V*, $R^1 = R^2 = COMe$) in various rheumatic conditions were also favourable[217,218].

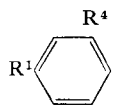


(*V*)

Michotte[219] has suggested that biological oxidation processes in mesenchymal tissues require a balance of C_{11}-hydroxysteroids and adrenergic substances, and this is disturbed in rheumatic diseases owing to the failure of the adrenergic component. It is alleged that pyrocatechoic acid (*V*) may replace this component, restore the physiological imbalance and, consequently, suppress the inflammatory reaction. Adams and Cobb[68] found that neither pyrocatechoic acid nor its diacetyl derivative administered orally inhibits ultra-violet erythema in the guinea-pig. The former compound, administered subcutaneously, reduces vascular permeability in the mouse peritoneum, but the effect is less than that of salicylic acid[208]. Other dihydroxybenzoic acids tested are not active in this system[175,208].

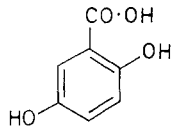
It has been suggested that hyaluronidase activity is increased in rheumatic

Table 2.3. Anti-inflammatory and analgesic activities of salicylamide and cresotinamide derivatives.
(*After* Gorini, Valcavi and Zonta-Bolego[214])

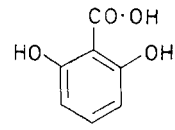


Compound number	R^1	R^2	R^3	R^4	LD_{50} i.p. mg/kg	*Anti-inflammatory activity*		*Analgesic activity*	
						Dose mg/kg i.p.	*Percentage inhibition*	*Dose* mg/kg i.p.	*Percentage activity*
1	$CO\cdot NH_2$	OH			660	215	33·30	135	43
2	$CO\cdot NH_2$	OEt			495	160	30·95	100	91
3	$CO\cdot NH_2$	$O\cdot CH_2\cdot CH{=}CH_2$			320	110	44·28	65	50
4	$CO\cdot NH_2$	$O\cdot CH_2\cdot COOH$			>4000	1000	54	1000	10
5	$CO\cdot NH_2$	$O\cdot CH_2\cdot CH{=}CHPh$			>2000	500	28	300	9
6	$CO\cdot NH_2$	$O\cdot CH_2\cdot CH{=}CHMe$			450	150	27	90	16·60
7	$CO\cdot NH_2$	$O\cdot CH_2\cdot CH{=}CH_2$		$MeCO\cdot NH$	440	184	28·55	104	38·33
8	H	$O\cdot CH_2\cdot CH{=}CH_2$		$MeCO\cdot NH$	480	160	62·32	100	70
9	$CO\cdot NH_2$	OH	Me		570	190	44·48	115	60
10	$CO\cdot NH_2$	$O\cdot CH_2\cdot CH{=}CH_2$	Me		420	140	45·12	85	84
11	$CO\cdot NH_2$	OEt	Me		510	170	27·83	100	23
12	$CO\cdot NH_2$	$O\cdot CH_2\cdot CH{=}CHPh$	Me		645	215	34·74	130	24
13	$CO\cdot NH_2$	$O\cdot CH_2CMe{=}CH_2$	Me		375	125	45·77	75	8
14	$CO\cdot NH_2$	$O\cdot CH_2\cdot CH{=}CHMe$	Me		625	205	40·58	125	44
15	$CO\cdot NH_2$	OH	Me	$CH_2{=}CH\cdot CH_2$	460	155	39	95	57
16	$CO\cdot NH_2$	OH	Me	$MeCO\cdot NH$	525	175	51	105	5

diseases[220], and that 2,5-dihydroxybenzoic acid (gentisic acid, *VI*), a metabolite of salicylate, is a potent inhibitor of the enzyme[221] (theories which are no longer tenable[169]). In view of this, gentisic acid was first used in rheumatic fever and rheumatoid arthritis with satisfactory results[204,222–226], but other workers found it an ineffective therapeutic agent[227–229].



(*VI*)



(*VII*)

In various other experimental inflammatory tests, gentisic acid proved to be active when administered intraperitoneally in one type[172] and inactive orally and subcutaneously in others[66,68,175,176,230]. It does not inhibit fibrinolysin *in vitro*[172].

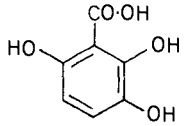
In the belief that a second chelate ring might enhance the activity of sodium salicylate, 2,6-dihydroxybenzoic acid (γ-resorcylic acid, *VII*) was tested in rheumatic fever and found to be effective in a dosage about one-tenth that of salicylate. There were many varied and undesirable side-effects. In a further trial, this compound was found to be five times more active than sodium salicylate in rheumatic fever, with fewer toxic reactions[204], but others claim that the drug has little or no effect[229,232]. The compound reduces formalin oedema in the mouse-foot in doses at which sodium salicylate is ineffective[233], and inhibits the formation of granulation tissue in artificial wounds in mice in doses similar to those in which cortisone is also effective although salicylate is not[234]. When administered intraperitoneally[172], it reduces anaphylactic arthritis in the guinea-pig but is ineffective in ultraviolet erythema in this species when given by mouth[66,68]. When given subcutaneously, it does not affect vascular permeability in the mouse[175], or formalin-induced mouse-foot oedema[176]. There is no *in vitro* inhibition of fibrinolysin[172].

2,4-Dihydroxybenzoic acid (β-resorcylic acid) is alleged to be as active as sodium salicylate in rheumatic fever[204], but it does not affect vascular permeability in the mouse[175]. 3,4-Dihydroxybenzoic acid (protocatechoic acid) is not active in rheumatic fever[204] and is barely effective in inhibiting anaphylactic arthritis in the guinea-pig[172]. When administered subcutaneously it does not reduce vascular permeability in the mouse[175], but reduces formalin-induced oedema in the mouse-foot[176]. When given intraperitoneally, 3,5-dihydroxybenzoic acid does not reduce yeast-induced oedema in the rat-paw[97], and subcutaneously does not affect vascular permeability in the mouse[175], or formalin oedema in the mouse-foot[176].

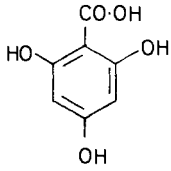
Trihydroxybenzoic acids

Clarke, Clarke and Mosher[204] claim that 2,3,6-trihydroxybenzoic acid (*VIII*) is 10 times, and 2,4,6-trihydroxybenzoic acid (*IX*) 8 times, more active than sodium salicylate in rheumatic fever, but Hamilton and Bywaters found (*IX*) to be inactive in three patients receiving 1–8 g in 24 hours[235].

Given intraperitoneally, (*IX*) reduces swelling in guinea-pig anaphylactic arthritis[172], but is inactive even in oral doses of 240 mg/kg in ultra-violet erythema in the same species. It is also inactive in a mouse vascular permeability test in subcutaneous doses of 200 mg/kg.[208]



(*VIII*)



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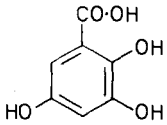

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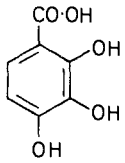
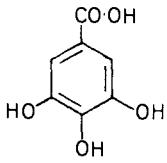


(*XI*)

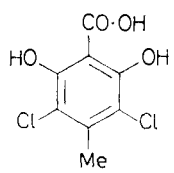


(*XII*)

It has been claimed that 2,3,5-trihydroxybenzoic acid (*X*) and its triacyl derivatives possess antihyaluronidase and anti-arthritic activities[236], but later work by the same authors suggests that they are inactive as analgesic or anti-arthritic drugs[237]. 2,3,4-Trihydroxybenzoic acid (*XI*) is inactive at 200 mg/kg subcutaneously and 3,4,5-trihydroxybenzoic acid (*XII*) is active at a subcutaneous dose of 118 mg/kg in a mouse vascular permeability test[208].

Substituted dihydroxybenzoic acids and dihydroxybenzenes

Several substituted dihydroxybenzoic acids have been examined by the yeast oedema and granuloma pellet test in rats[97], and anti-inflammatory activity appears to be restricted to the substituted 2,6-dihydroxy compounds. The presence of a halogen at C_3 and C_5 produces activity which is further increased when a methyl or benzyl group is also present at C_4. 4-Benzyl-2,6-dihydroxybenzoic acid is also active, despite the absence of halogen at C_3 or C_5. The most potent compound of all is 3,5-dichloro-2,6-dihydroxy-4-methylbenzoic acid (*XIII*). Whenever anti-inflammatory activity has been



(*XIII*)

found, the doses used have been near the toxic levels, and attempts to separate the two components have not been successful. A comparison of the active doses (subcutaneous for the pellet assay and intraperitoneal for the oedema

tests) and lethal doses (*Table 2.4*) suggest that some of the activity may be a manifestation of toxicity rather than of genuine anti-inflammatory action.

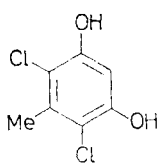
Table 2.4. Anti-inflammatory activity in a series of substituted dihydroxybenzoic acids

Name	mg/kg	*Activity*		*Approximate* LD50 *i.v. mouse* (mg/kg)
		Granuloma pellet test	*Rat-paw oedema test*	
2,6-Dihydroxy-4-methylbenzoic acid	150	0	+	380
3,5-Dibromo-2,6-dihydroxybenzoic acid	300		+	350
3,5-Dibromo-2,6-dihydroxy-4-methylbenzoic acid	150	0	+	138
3,5-Dichloro-2,6-dihydroxy-4-methylbenzoic acid	150	+ (P=0·001)	+	125
2,6-Dihydroxy-4-(hydroxymethyl)benzoic acid	600		0	1200
4-Benzyl-2,6-dihydroxybenzoic acid	150		+	100
3-Benzyl-2,6-dihydroxybenzoic acid	75		+	
2,6-Dihydroxy-4-(*p*-tolylmethyl)benzoic acid	37·5		+	45
4-Benzyl-3,5-dibromo-2,6-dihydroxybenzoic acid	75		+	105
3,5-Dichloro-2,6-dihydroxy-4-methylbenzamide	75		+	110
2,4-Dihydroxy-5-methylbenzoic acid	150	0	0	545
5-Chloro-2,4-dihydroxybenzoic acid	150	0	0	1075
3,5-Dichloro-2,4-dihydroxybenzoic acid	150	0	0	1000
2,4-Dihydroxy-3-methylbenzoic acid	300		0	780
5-Benzyl-2,4-dihydroxybenzoic acid	150		0	
4,5-Dibromo-2,3-dihydroxybenzoic acid	75		0	75
5-Chloro-2,3-dihydroxybenzoic acid	150		0	
5-Bromo-3,4-dihydroxybenzoic acid	150		0	180
3,5-Dihydroxybenzoic acid	150		0	2000
4-Chloro-3,5-dihydroxybenzoic acid	150		0	2000
3-Chloro-4-methylbenzoic acid	160	+ (P=0·01)	+	208

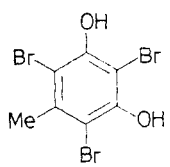
(*From* Lightowler and Rylance[97], *by courtesy of* Journal of Pharmacy and Pharmacology, London)
0 Ineffective at the highest tolerated dose.
+ Effective, swelling less than 20 per cent.

This view is supported by the experiments of Smith, Frommel and Radouco-Thomas[238] who found (*XIII*) to be lethal in guinea-pigs at the dose at which it suppresses ultra-violet erythema.

In view of the activity of compound (*XIII*), Lightowler and Rylance[239] examined a number of similar compounds without the carboxyl group.



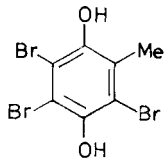
(*XIV*)



(*XV*)

The compound corresponding to (*XIII*), 4,6-dichloro-5-methylresorcinol (*XIV*) shows some anti-inflammatory activity, whilst other halogenated 5-methylresorcinols (e.g. *XV*) are also active. It was found that the resorcinol nucleus is the best structure for activity, since the bromo derivatives from 2-methylhydroquinone (*XVI*) and from 4-methylcatechol (*XVII*) are

inactive. The importance of the two hydroxyl groups is demonstrated by the fact that compound (*XVIII*) is inactive.



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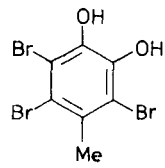
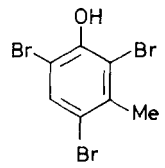


(*XVII*)



(*XVIII*)

In this series, as with the substituted dihydroxybenzoic acids, toxicity and activity cannot be divorced.

Cresotinic acids

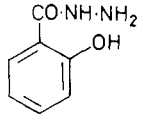
The ortho-, meta- and para-cresotinic acids were originally claimed by Stockman[180,205] to possess a powerful specific action in acute rheumatic conditions, though having no advantages over salicylic acid. All three compounds possess anti-inflammatory activity in the mouse[175,208].

Miscellaneous compounds related to salicylic acid

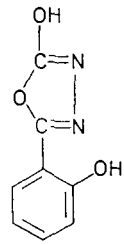
Northover[208] investigated the effects of a miscellaneous group of hydroxybenzoates and related compounds in a mouse vascular permeability test. He concludes that the simplest active compound is salicylic acid, that the carboxyl group is essential for activity and that, whilst one hydroxyl group adjacent to the carboxylic acid group confers activity, further hydroxyls either abolish or reduce the effects. Halogenated congeners, 5-chloro- and 3,5-di-iodosalicylic acid are active but toxic. Our own unpublished studies on the activities of hydroxybenzoates in ultra-violet erythema are in general agreement with Northover's conclusions. 4-Aminosalicylic acid does not possess anti-inflammatory activity in animal tests[66,68,175].

Some hydroxycinnamates have been examined for their ability to reduce vascular permeability in the mouse peritoneum[208]; as in the benzoic acid series, activity was found in the 2-hydroxy but not in the 3- and 4-hydroxycinnamates.

In the belief that the gastric irritation produced by salicylates may be partly due to the free carboxyl group, Smith, Frommel and Radouco-Thomas[238] prepared the corresponding hydrazide (*XIX*) and oxadiazol-2-ol (*XX*) of salicylic acid.



(*XIX*)

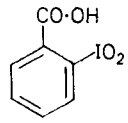


(*XX*)

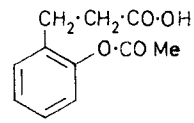
Of a number of oxadiazole derivatives of other substituted benzoic acids tested, most possess analgesic and antipyretic activities, compound (*XX*) being similar to acetylsalicylic acid; none shows any anti-inflammatory activity.

The calcium salt of 2-iodoxybenzoic acid (*XXI*) administered in a dose of 1·5 to 6 g daily to a group of arthritic patients was found to produce a definite improvement in 71 per cent[240].

β-(2-Acetoxyphenyl)propionic acid (*XXII*) possesses analgesic properties and reduces silver nitrate-induced inflammation and swelling in the joints of rats. In a limited trial, six arthritic patients seemed to experience some relief of joint pain, but all suffered side-effects[241].

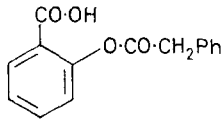


(*XXI*)

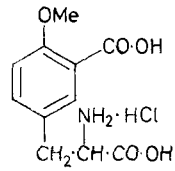


(*XXII*)

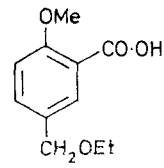
o-(Diphenylacetoxy)benzoic acid has superior analgesic but weaker antipyretic properties than acetylsalicylic acid, but is ineffective in the cotton-pellet granuloma test in rats[242]. It has been claimed that 5-acetylglycolloylsalicylic acid and its methyl ester possess anti-inflammatory and anti-arthritic properties[243]. At a dose of 250 mg/kg orally, chloroquine 4-acetamidosalicylate inhibits both viscarin-induced oedema and cellular colonization of Ivalon pellets in rats[244]. Lespagnol and Thiéblot found that phenacetylsalicylic acid (*XXIII*), like sodium salicylate, reduces the permeability of rabbit synovial membrane[245].



(*XXIII*)



(*XXIV*)

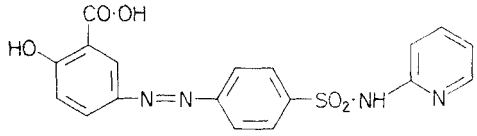


(*XXV*)

Two derivatives GPA69 (*XXIV*) and GPA415 (*XXV*) show an analgesic activity superior to that of acetylsalicylic acid in animal tests, but are devoid of antipyretic and anti-inflammatory activities[246]. Both are analgesics in man when measured by the electrical tooth-stimulation technique, a direct form of pain without an inflammatory component. In trials in different types of clinical pain, however, their effects vary, negative results being obtained in conditions in which pain has a significant inflammatory component. These results imply that the value of acetylsalicylic acid in relieving pain is partly attributable to its anti-inflammatory effects and partly to other peripheral effects.

Sulphasalazine, 4-hydroxy-4′-(*N*-pyrid-2-ylsulphamoyl)azobenzene-3-carboxylic acid (sulphasalazine, Salazopyrin, *XXVI*), was developed from

the work of Svartz in the late 1930s when chemical compounds of salicylic acid and sulphonamides were produced for use in rheumatoid arthritis. This drug, like other acid azo-compounds, has a specific affinity for connective tissues, especially those rich in elastin[247]. Thus, depots are formed in the connective tissue and subsequent splitting of the molecule releases 5-aminosalicylic acid and sulphapyridine[248]. Svartz believed that, since the lesion of rheumatic disease is inflammatory in nature and located in connective tissue, drugs with an affinity for connective tissue were likely to be useful. Sulphasalazine produces satisfactory results in rheumatic fever and rheumatoid arthritis, particularly when treatment is continued for periods of from several months to 2 or 3 years[248–252]. Sinclair and Duthie[253], in a later trial, concluded that sulphasalazine appears to be of no specific value in the treatment of rheumatoid arthritis.

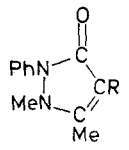


(XXVI)

It is of interest that later work indicates that 5-aminosalicylic acid itself has an affinity for connective tissue[254–256]. It is nevertheless difficult to conceive that 5-aminosalicylic acid could be effective, even if it does concentrate in connective tissue, since there is no experimental evidence that this compound has anti-inflammatory activity. We have ourselves found it to be inactive in the ultra-violet erythema test in guinea-pigs.

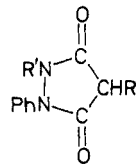
Pyrazolones and Related Compounds

It is convenient to divide the anti-inflammatory pyrazolones into two groups: pyrazol-5-ones (*XXVII*), typified by amidopyrine, and pyrazolidine-3,5-diones (*XXVIII*), of which the principal member is phenylbutazone.



R=H, alkyl, aryl or alkylamino group

(XXVII)



R= alkyl, substituted alkyl or alkoxy group
R'= aryl or substituted aryl group

(XXVIII)

Pyrazol-5-ones

The best known members of this group are phenazone (antipyrine,

XXVII, R = H) and amidopyrine (*XXVII*, R = NMe_2), which has long been used as an analgesic, antipyretic and antirheumatic agent. A recent controlled study confirmed the activity of amidopyrine in rheumatoid arthritis[186]. In recent years, however, its clinical use has declined considerably owing to growing recognition of its dangerous side-effects, in particular agranulocytosis. The history of the use of amidopyrine has been reviewed and its judicious use defended[257]. Most of the information on the anti-inflammatory activity of amidopyrine has come from relatively recent studies by workers who, using modern techniques, have frequently employed amidopyrine as a reference compound with which to compare newer anti-inflammatory agents.

Against ultra-violet erythema in guinea-pigs, amidopyrine has an oral ED50 of 130 mg/kg and is roughly equal in effect to acetylsalicylic acid and has one-seventh of the potency of phenylbutazone[66]. This finding was substantially confirmed by Adams[158], who found amidopyrine to be effective at 80 mg/kg, this being equal to the effective dose of acetylsalicylic acid and one eighth that of phenylbutazone. Wilhelmi and Domenjoz found it active in a dose of 40 mg/kg subcutaneously[258]. Domenjoz[113] found that, in oedema tests, amidopyrine performs better than phenylbutazone, 200 mg/kg inhibiting formalin, serotonin and dextran oedema to the extent of 30, 58 and 74 per cent, respectively. However, in a critical study[105] of the effect of anti-inflammatory substances in various rat-paw oedema tests, amidopyrine was found to be inactive in the formalin test and appreciably less active than phenylbutazone in the dextran and serotonin tests. In a dose of 125 mg/kg it suppresses kaolin oedema more than does phenylbutazone at 50 mg/kg, both drugs being given intraperitoneally. Amidopyrine is appreciably less effective than phenylbutazone in the granuloma-pouch test[113]. Smith, Parsons and Whitehouse[259] found that, in a daily oral dose of 200 mg/kg, amidopyrine failed to reduce cotton-pellet granuloma formation.

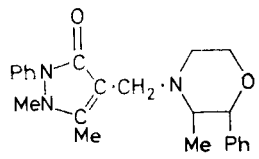
Several analogues of amidopyrine have been prepared, differing from the parent compound mainly in the substituent at C_4 of the pyrazole ring. Dipyrone (Novalgin, *XXVII*, R = $NMe \cdot CH_2 \cdot SO_3^-Na^+$), a water-soluble analogue of amidopyrine is rather more active than the parent compound in ultra-violet erythema and of similar activity in croton oil inflammation in mice[258]. It also shows similar activity to amidopyrine in kaolin oedema of the rat-paw[260]. 2,3-Dimethyl-4-isopropyl-1-phenylpyrazol-5-one (Isopyrin, *XXVII*, R = $NH \cdot CHMe_2$) is similar to amidopyrine in its anti-inflammatory activity as judged by rat-paw tests[261]. Its analgesic activity is also similar to that of amidopyrine though its antipyretic potency is lower. It is a sedative and, in this and certain other general pharmacological properties, differs from amidopyrine.

Propylphenazone (*XXVII*, R = $CHMe_2$) has an analgesic potency similar to that of amidopyrine[262] but its anti-inflammatory activity is reported to be less than half[260].

2,3-Dimethyl-4-(3-methyl-2-phenylmorpholino) methyl-1-phenylpyrazol-5-one (Tarugan, *XXIX*) is reported to have 2–3 times the analgesic activity of amidopyrine and twice its activity in dextran oedema of the rat-paw[263].

By incorporating nicotinamide into the side chain at C_4 a potent analgesic drug[264] with reduced toxicity was obtained (Nifenazone, Nicopyron,

Thylin, *XXVII*, R = NH·CO— ). The anti-inflammatory activity of the compound was demonstrated by Hiemeyer[265] who showed it to be effective in reducing the duration of erythema produced in volunteers by the intracutaneous injection of bacterial lipopolysaccharide. The compound,

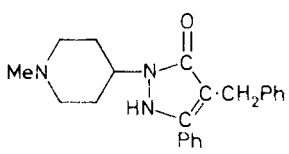


(*XXIX*)

however, proved ineffective in controlling the manifestations of rheumatoid arthritis[266].

The toxicological and pharmacological properties of 2,3-dimethyl-4-(*N*-nicotinamidoacetamido)-1-phenylpyrazol-5-one hydrochloride (NAI, *XXVII*, R = NH·CO·CH$_2$·NH·CO— ), an injectable modification of Nicopyron, have been described[267]. Although it possesses low acute and chronic toxicity, it is also much less active than amidopyrine and phenylbutazone in oedema and granuloma tests. Favourable clinical results are reported[268].

Hart and Boardman[269] tested 4-benzyl-1-(1-methyl-4-piperidinyl)-3-phenyl-3-pyrazolin-5-one (KB95, *XXX*) and found no evidence of anti-inflammatory activity, though a few patients appeared to have symptomatic relief.



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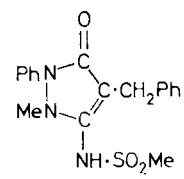


(*XXXI*)

Bucher[270] has described a test for anti-inflammatory compounds which involves the intraperitoneal injection of talc into guinea-pigs to produce peritonitis. One of the behavioural manifestations of this is inhibition of gnawing activity, which is restored to normal by various analgesic and anti-pyretic substances, especially pyrazolones. Phenylbutazone and amidopyrine are both active in doses as low as 5 mg/kg subcutaneously. Acetylsalicylic acid is also active, but prednisolone affects the response only when given in high doses. This test was used to demonstrate anti-inflammatory activity in a series of pyrazolones in which the usual 3-methyl group is replaced by alkylsulphonamido radicals[271]. The most active compound (*XXXI*) is equal in efficacy to phenylbutazone.

Pyrazolidine-3,5-diones

Phenylbutazone (Butazolidine, *XXVIII*, R = Bu, R^1 = Ph) is apparently the most active and certainly the most thoroughly investigated anti-inflammatory compound of the pyrazole series. It was first synthesized in 1946 by Stenzl[272] and is the subject of a recent monograph[273].

Phenylbutazone is active in a wide range of acute inflammatory reactions, including ultra-violet and heat-induced erythema in the guinea-pig and inflammatory oedema produced by various agents in the skin and paws of the guinea-pig, rat and rabbit[273]. It is very active in suppressing thurfyl nicotinate erythema in guinea-pigs, 30 per cent suppression of the response being achieved with an oral dose of 5 mg/kg[12]. Similar activity is shown against ultra-violet erythema[12,158]. Sharp[150] demonstrated that phenylbutazone, when given daily in doses of 16 mg/kg by intraperitoneal injection, reduces synovial permeability in the rabbit.

In various rat-paw oedema tests the dose of phenylbutazone necessary to produce a response is much higher than that required to reduce erythema. For example, doses of 100–200 mg/kg are required to reduce formalin oedema; this is up to 30 times the maximal clinical dose.

A quantitative difference exists in the responsiveness of various types of oedema[274]; formalin oedema responds more readily than dextran oedema in the same animal. Lorenz[103] found kaolin oedema in rats to be far more susceptible than egg-white or formalin oedema to phenylbutazone in oral doses of 3 to 50 mg/kg. Wagner-Jauregg, Jahn and Büch[260] confirmed that kaolin oedema is the most sensitive of the rat-paw tests. In their hands, 100 mg/kg of phenylbutazone orally inhibited swelling by 36 per cent. Cohen and Got[275], giving phenylbutazone intraperitoneally in a dose of 100 mg/kg, obtained a very marked response in kaolin oedema, and found, moreover, that the response is less at low room temperatures. Varga, Méhes, Pár and Rónai[105] showed that phenylbutazone in a dose of 50 mg/kg intraperitoneally inhibits oedema of the rat's paw caused by kaolin, dextran, 5-hydroxytryptamine and formalin.

Various effects of phenylbutazone on inflammatory cells have been described[273]. Antweiler[276] showed suppression by doses of 50 to 100 mg/kg of the phagocytic activity of blood leucocytes. Studer and Bächtolder[277] found little effect on the extravascular distribution or intravascular accumulation of leucocytes. Evidence of a reduced cell content of exudate in the acute response to a cotton-wool pellet is furnished by Saxena[47], who gave 200 mg/kg intramuscularly, a dose which failed to affect the volume of exudate.

Despite some discordant data[278], it seems that phenylbutazone does not affect the tuberculin reaction in BCG-infected guinea-pigs[136,279].

Unlike the salicylates, phenylbutazone shows definite activity in various granuloma tests[273], for example in the cotton-pellet test over a dose range of 33–100 mg/kg[280]. The slope of the dose response curve is much less steep than that of the corticosteroids. On the other hand, Winter, Risley and Nuss[112] did not find such a difference. Granuloma resulting from the intraperitoneal injection of silica in rats is inhibited by up to 40 per cent by about 50 mg/kg of phenylbutazone[281]. Trnavský, Trnavská and Malinský[282], on observing the effect of phenylbutazone on the morphology and biochemical components of

a developing turpentine granuloma in rats, found that phenylbutazone suppresses the early cellular reaction but does not interfere with collagen synthesis. There is, perhaps, an increase in collagen maturation. The authors therefore consider that phenylbutazone comes into the category of anti-permeability, anti-exudative agents and not the anti-proliferative groups typified by corticosteroids.

In various analgesic tests phenylbutazone is active, though less so than amidopyrine[113]. Clinically, however, it is of little value as an analgesic in pain of non-inflammatory origin[246]. Randall and Selitto[283] demonstrated a clear cut analgesic effect in inflammatory pain as measured by tolerance of pressure applied to the yeast-inflamed foot, a result which was confirmed by Crepax and Silvestrini[284].

There is evidence of analgesic activity on the part of phenylbutazone, in that a mean dose of 32 mg/kg intravenously antagonizes the pseudaffective response of dogs to injected bradykinin[285]. Amidopyrine was not active in this test. Later investigations by the same workers[286] indicate that phenylbutazone is a peripherally active analgesic rather than a member of the group of centrally acting drugs which includes the narcotics.

The clinical use of phenylbutazone in the treatment of rheumatoid arthritis, other rheumatic diseases, thrombophlebitis and many other inflammatory conditions has been reviewed[273].

Oxyphenbutazone

Oxyphenbutazone (Tanderil, G27202, *XXVIII*, R = Bu, R′ = *p*-HO·C_6H_4) is a metabolite of phenylbutazone[287,288], which it resembles in some of its anti-inflammatory properties. Against ultra-violet erythema in guinea-pigs some workers have found it inactive[11,113] while others[66] have found that, in a dose of 50 mg/kg, it has an anti-erythemic effect equal to that of 18 mg/kg of phenylbutazone. In formalin oedema of the rat-foot, oxyphenbutazone is similar in activity to phenylbutazone[13,289] but in 5-hydroxytryptamine and dextran induced swellings the metabolite is practically inactive. It is 2–3 times as effective as phenylbutazone in the kaolin oedema test[260].

In the granuloma-pouch test, 100 mg/kg daily of oxyphenbutazone reduces the granuloma weight by 50 per cent and the exudate by 75 per cent; it is therefore much more active than phenylbutazone[113]. It is also more active than phenylbutazone as an antipyretic but is practically inactive as a central analgesic[289].

A clinical comparison of oxyphenbutazone with phenylbutazone has shown that the two drugs are similarly effective and that the incidence of side effects is the same[290]. Other controlled trials of oxyphenbutazone have also been reported[291–293]. As with amidopyrine, prolonged use of phenylbutazone and oxyphenbutazone has been restricted by the fear of dangerous side effects, particularly those on the blood and haemopoietic organs[294,295].

Many modifications of the phenylbutazone structure have been made in attempts to increase activity and reduce toxicity. When the butyl group is replaced by propyl or allyl, almost all the anti-inflammatory activity of phenylbutazone is retained[67]. Activity also persists when the C_4 of one or both benzene rings carries substituents including methyl, carboxyl[67],

chloro[296] or nitro groups[297]. The nitro compound is of interest since, unlike some other analogues, it has anti-inflammatory, sodium retaining and uricosuric effects[297]. Substitution by a methyl group of the hydrogen atom at C_4 of the pyrazolidine ring destroys anti-inflammatory activity, suggesting that an enolizable 1,3-dioxo system is necessary[67]. In agreement with this, the methyl ether of the enolic form is inactive[298]. Other workers have, however, found activity in phenylbutazone analogues in which this criterion is not fulfilled[299]. Compounds in which cyclopentane or cyclopentene rings take the place of the pyrazolidine ring are also inactive.

Monophenylbutazone (4-*N*-butyl-1-phenylpyrazolidine-3,5-dione) has anti-inflammatory activity in various rat-paw oedema tests and exerts a beneficial effect on joint size and on the erythrocyte sedimentation rate in patients with rheumatoid arthritis[300]. Earlier work by Franchimont, Lecomte and van Cauwenberge[301] shows monophenylbutazone to be inactive in four different oedema tests; the dose investigated, however, was rather small (10 mg/kg).

Phenylbutazone analogues with modified butyl side-chain

When metabolized, phenylbutazone yields two hydroxylated derivatives[296]. The first is oxyphenbutazone, the properties of which are described above; the second contains an alcoholic hydroxyl group in the butyl side-chain (*XXVIII*, R = $(CH_2)_2 \cdot CHOHMe$, R′ = Ph). This compound is devoid of anti-inflammatory activity but has enhanced uricosuric potency. Other derivatives with a modified side-chain show increased uricosuric activity associated with anti-inflammatory action. The most important are the phenylthioethyl analogue (G25671, *XXVIII*, R = $(CH_2)_2 \cdot SPh$, R′ = Ph) and its metabolite sulphinpyrazone (Anturan, *XXVII*, R = $(CH_2)_2 \cdot SOPh$, R′ = Ph).

Compound G25671 has analgesic activity of the same order as phenylbutazone when tested by the rabbit dental-pulp method, both compounds being less active than amidopyrine[93,113], and is superior to phenylbutazone as an antipyretic. The metabolite of compound G25671, sulphinpyrazone, is inactive in both respects.

In rat-paw oedema tests both compound G25671 and sulphinpyrazone are active. Sulphinpyrazone is particularly active against formalin oedema and, in a dose of 300 mg/kg orally, achieves 92 per cent inhibition, a result far superior to that for any other pyrazolidine derivative tested. In 5-hydroxytryptamine oedema, compound G25671 is inactive, whilst sulphinpyrazone exceeds phenylbutazone in activity. Both the sulphur analogues reduce dextran oedema, against which phenylbutazone and oxyphenbutazone have very low activity. Compound G25671 is somewhat more active than sulphinpyrazone, and slightly less active than phenylbutazone, in the Selye granuloma-pouch test.

Ultra-violet erythema in guinea-pigs is retarded by both compounds, G25671 being about equal in activity to phenylbutazone whilst sulphinpyrazone has only one-quarter of its activity. Compound G25671 inhibits ultra-violet erythema, the oral ED_{50} lying between 25 and 50 mg/kg.

Compound G25671, like phenylbutazone, exerts an anti-inflammatory effect in rheumatoid arthritis[93,302]. It differs from phenylbutazone, however,

in having a very short half-life in human plasma (3 hours compared with about 70 hours), in being devoid of sodium retaining properties, and in its enhanced uricosuric effect[302].

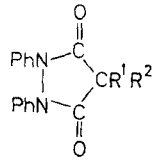
Phenylbutazone analogues with side-chain modifications which increase the acidity of the molecule have lower anti-inflammatory activity and sodium retaining potency but an increased uricosuric effect[303]. As would be predicted on this basis, a keto analogue of oxyphenbutazone (G29701 *XXVIII*, $R = CO \cdot (CH_2)_2Me$, $R' = p\text{-}HO \cdot C_6H_4$) has no anti-inflammatory effect but has a uricosuric potency comparable to that of sulphinpyrazone[304]. On the other hand, a keto analogue of phenylbutazone (Ketazon, *XXVIII*, $R = CH_2 \cdot CH_2 \cdot COMe$, $R' = Ph$) is reported to have anti-inflammatory activity[305], and is used as an antirheumatic drug in Czechoslovakia. Its cyanacethydrazone apparently has greater activity and lower acute toxicity[306].

In various oedema tests and in the cotton-wool granuloma test in rats, 4-(3¹-chlorocrotyl)-1,2-diphenylpryazolidine-3,5-dione (*XXVIII*, $R = CH_2 \cdot CH{:}CClMe$, $R' = Ph$) had anti-inflammatory activity of about the same magnitude as phenylbutazone, both being tested in doses of 100–200 mg/kg[307]. The analogue is said to have lower acute and chronic toxicity than the parent compound.

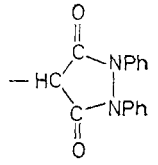
In a series of 4-substituted 1,2-diphenylpyrazolidine-3,5-diones (*XXVII*, R = alkyl, aryl, aralkyl, etc., $R' = Ph$) many showed analgesic, anti-inflammatory and antipyretic activity. The lauryl, α-methylethylidene and furfurylidene compounds are reported to be more active than phenylbutazone though less so than its calcium salt[308].

Miscellaneous analogues of phenylbutazone

Using various rat-paw oedema tests, Piccinini, Marazzi-Uberti and Lugaresi[309] found that 1,2-diphenylpyrazolidine-3,5-dione (*XXXII*, $R^1 = R^2 = H$) is inactive but that the bi- and tri- compounds (*XXXII*, $R^1 = H$, $R^2 = XXXIII$, and *XXXII*, $R^1 = R^2 = XXXIII$) are 1–2 and 3–5 times respectively, more active than phenylbutazone.



(*XXXII*)

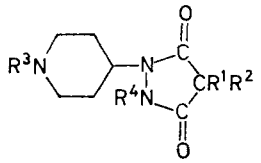


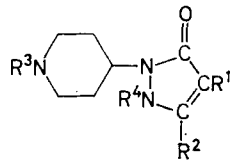
(*XXXIII*)

A series of substituted 1,3-diarylbarbituric acids has been tested for anti-inflammatory activity in the Randall and Selitto and pleural effusion tests. Some members are active but none approaches the potency of phenylbutazone[310].

A Swiss team[299,311] has investigated several basic-substituted pyrazolidinediones (*XXXIV*, R^1 and R^2 = a hydrocarbon group, R^3 = lower alkyl, R^4 = H, lower alkyl, acyl or carbamoyl). In the kaolin oedema test,

several show striking activity and one (*XXXIV*, $R^1 = R^2 = Bu$, $R^3 = Me$, $R^4 = Pr^i$) is more active than phenylbutazone. Starting from basic-substituted hydrazines, Jucker[311] prepared a series of substituted pyrazol-5-ones of general structure *XXXV*. Several of these compounds have anti-inflammatory



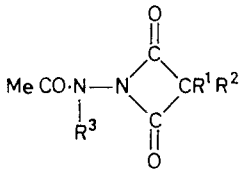


R^1 and R^2 = lower alkyl, aryl or aralkyl

R^3 = lower alkyl, R^4 = H or lower alkyl

(XXXIV) *(XXXV)*

activity when tested against formalin and 5-hydroxytryptamine oedema in rats. Pharmacological details are not given and the results of testing by other methods would be interesting. Activity is also retained in the 5-amino-



R^1 and R^2 = lower alkyl

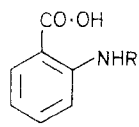
R^3 = usually *N*-alkylpiperidyl

(XXXVI)

pyrazoles and in certain azetidin-2,4-diones (*XXXVI*), some of the latter having activity also in the granuloma-pouch test[311].

N-*Arylanthranilic Acids*

These compounds, derived from anthranilic acid (*XXXVII*, R = H), can be considered as analogues of salicylic acid. Three active anti-inflammatory



(XXXVII)

N-substituted anthranilic acids, mefenamic acid (*N*-2,3-xylylanthranilic acid, Ponstan, *XXXVII*, R = 2,3-xylyl)[127], flufenamic acid (*N*-(α,α,α-trifluoro-*m*-tolyl)anthranilic acid, *XXXVII*, R = 3-trifluoromethylphenyl)[280] and CI-583 (*XXXVII*, R = 2,6-dichloro-3-methylphenyl)[312] have been described.

The anti-inflammatory activity of mefenamic acid by oral administration is 51 per cent of that of phenylbutazone, determined by the ultra-violet erythema assay in guinea-pigs. When tested by the subcutaneous cotton-wool pellet method, the antigranulation effect of mefenamic acid is 60 per cent of that of phenylbutazone, both compounds producing shallow dose effect slopes. It is claimed that mefenamic acid differs from flufenamic acid in possessing a moderate degree of central analgesic activity, thus resembling amidopyrine and acetylsalicylic acid rather than phenylbutazone and oxyphenbutazone. Mefenamic acid also possesses marked antipyretic activity. The anti-inflammatory activity (ultra-violet erythema) of flufenamic acid is 3·2 times that of mefenamic acid and 16 times that of acetylsalicyclic acid. It is an active antipyretic drug, but its antipyretic anti-inflammatory ratio is lower than that of acetylsalicylic acid, amidopyrine or mefenamic acid. In the rat cotton-wool pellet test, it has an oral potency 3·6 times that of phenylbutazone. It lacks the analgesic effect of mefenamic acid when tested by a tail-pinch method in rats, and may not, therefore, be effective in the relief of pain unless there is an underlying inflammatory cause.

N-(2,6-Dichloro-3-methylphenyl)anthranilic acid (CI-583) is the most active anthranilic compound yet reported[312]. As an anti-inflammatory agent, it is 15 times more potent than phenylbutazone and 150 times more potent than acetylsalicylic acid. Laboratory results suggest that its side-effects may be less than those of mefenamic or flufenamic acids. According to Scherrer[312], the most active anthranilic acids are those which have substituents in the 2-, 3- and 6-positions of the ring which is attached to the anthranilic acid nitrogen atom. The most active disubstituted compounds are the 2,3-derivatives. Lespagnol and Thieblot[245] found anthranilic acid (*XXXVII*, R = H), *N*-acetylanthranilic acid (*XXXVII*, R = MeCO) and phenacetylanthranilic acid (*XXXVII*, R = phenacyl) inactive in the rabbit synovial membrane test. In mouse peritonitis, a reduction of vascular permeability is produced by subcutaneously-administered mefenamic and flufenamic acids, but not by anthranilic acid[208]. In the guinea-pig, intravenous doses of mefenamic and flufenamic acids antagonize bronchoconstriction produced by bradykinin and kallidin-10 in doses similar to effective intravenous doses of acetylsalicylic acid[313].

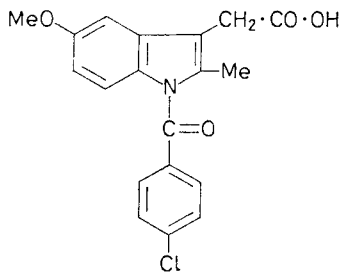
Absorption of mefenamic acid in animals takes place in the small intestine; in man, peak plasma levels of nearly 1 mg/100 ml are reached within 2 to 3 hours of oral administration. Excretion of mefenamic acid and its two principal metabolites is mainly renal; less than 5 per cent is excreted in the bile. Neither metabolite has significant analgesic or anti-inflammatory actions.

In clinical studies, Cass and Frederik[314] showed that mefenamic acid is 2–3 times more active than acetylsalicylic acid in relieving chronic pain. Over a 10 week period, flufenamic acid in a dosage of 300 mg daily is more effective than 1,800 mg a day of acetylsalicylic acid in rheumatoid arthritis[315].

Indol-3-ylalkanoic Acids

The most studied member of this series is 1-(4-chlorobenzoyl)-5-methoxy-2-methylindol-3-ylacetic acid (indomethacin, Indocid, *XXXVIII*) the synthesis of which has been described[316]. A large number of analogues, including

higher alkanoic acids, have also been reported[317–320], but no data on biological activity are available for these. It is claimed that substantially all the anti-inflammatory activity resides in the (+)-enantiomorph[321]. De-acylation gives essentially inactive compounds. The substitution of benzyl for benzoyl reduces activity slightly, whilst 5-demethylation reduces it more markedly.



(*XXXVIII*)

When 4-methylmercapto is substituted for 4-chloro, and 2-propionic acid for acetic acid, compounds less active than indomethacin are produced[322]. Other structural modifications have been discussed by Shen[323].

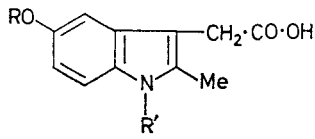
Indomethacin is a potent anti-inflammatory and anti-pyretic compound. Given orally, it inhibits the formation of the cotton-pellet granuloma in the rat, being about 85 times more potent than phenylbutazone, and equally active in both intact and adrenalectomized rats. It inhibits oedema of the rat-foot, produced by either carrageenan or mustard, but does not inhibit that produced by egg-white, formalin, yeast or 5-hydroxytryptamine. As an antipyretic, it is 10–20 times more powerful than phenylbutazone[112]. Indomethacin is well absorbed in all species, but there is considerable variation in its distribution and metabolism. Man tolerates the drug better than do other species, and is less liable to the gastro-intestinal irritation encountered in experimental animals[324].

After intravenous administration, 46–63 per cent of radioactive indomethacin is rapidly excreted in the bile of dogs, guinea-pigs and monkeys. Radioactive material in the bile is largely reabsorbed in the intestine of the guinea-pig and monkey, but not of the dog[325]. Renal clearance of the compound and its metabolites is the important excretory route in most species, except in the dog in which faecal excretion is high and urinary clearance negligible[326].

In the urine of laboratory animals, most of the drug is accounted for as unchanged indomethacin and its *O*-demethyl (*XXXIX*, R = H; R′ = *p*-$ClC_6H_4 \cdot CO$) and *N*-dechlorobenzoyl (*XXXIX*, R = Me; R′ = H) derivatives and the corresponding acyl glucuronides[326]. Conjugation and renal excretion occur increasingly in the sequence: rat, rabbit, guinea-pig and monkey. In man, there is no evidence of metabolic breakdown and almost all the urinary excretion is as the glucuronide.

Indomethacin is effective in controlling pain and stiffness in rheumatoid

arthritis and is also of value in gout, ankylosing spondylitis and osteoarthritis[327–331]. There is a high incidence of headache, dizziness and dyspepsia, and 29 per cent of patients withdrew from the trial reported by Percy, Stephenson and Thompson[329]. Gastro-intestinal side-effects are important and the drug causes gastro-intestinal bleeding (though less than that caused by acetylsalicylic acid) and peptic ulcers[328,332]. Most of the side-effects occurred with doses of 250–600 mg daily, but since the more conservative dosage regime of 25 mg twice or four times daily in capsules instead of



(XXXIX)

tablets has been used, the incidence of all side-effects has dropped from over 50 per cent to around 20 per cent[324].

Other substituted alkanoic acids and related compounds

Some alkanoic and alkenoic acids derived from biphenyl, stilbene and diphenylethane[333] were reported to have potential antirheumatic and hypocholesterolaemic properties. Some of these compounds were later investigated by Milla and Grumelli[334] for anti-inflammatory activity; the only compound which showed activity was 3-(4-biphenylyl)acrylic acid, *p*-$PhC_6H_4 \cdot CH = CH \cdot CO \cdot OH$. Some of the alkylamine esters of this acid prepared by Carissimi[335], and the acid itself, were then examined for anti-inflammatory activity in dextran oedema of the rat-foot, experimental granuloma in the rat, and synovial permeability in the rabbit, and for their ability to modify the reactions of subcutaneous connective tissue in mice.

Only 3-(4-biphenylyl)acrylic acid and its diethylaminoethyl ester, administered intraperitoneally, were active in dextran oedema, and both were also effective in synovial permeability. Both compounds also inhibit granuloma formation, but in this reaction, and contrary to the oedema tests, all the basic esters are found to exert an anti-granuloma effect when given intraperitoneally. The parent acid also reduces the disappearance of fibroblasts and the invasion by polymorphonuclear cells and macrophages in the inflamed connective tissue of mice. The parent acid possesses neither analgesic nor antipyretic activity.

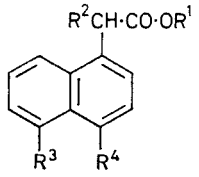
Further experiments with the basic esters of the unsaturated acid showed that they do not exert a direct antiproliferative effect on the growth of cultures of human tumour cells[336].

Bismuth biphenylylacrylate, in 5 mg suppositories, is effective in miscellaneous non-rheumatic inflammatory states such as acute tonsillitis, pharyngitis, sinusitis and adnexitis[337].

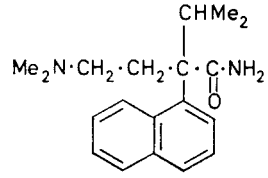
High anti-inflammatory activity has also been claimed for derivatives of 1-naphthyl-acetic and -propionic acids of the general formula (*XL*), of which the preferred compounds are 4-phenyl-1-naphthylacetic acid (*XL*,

$R^1 = R^2 = R^3 = H$, $R^4 = Ph$) and 5-phenyl-1-naphthylacetic acid (*XL*, $R^1 = R^2 = R^4 = H$, $R^3 = Ph$)[338], and also certain substituted α-naphthylacetic acid derivatives[339].

A wide range of anti-inflammatory activities has been claimed for α-(2-dimethylaminoethyl)-α-isopropyl-1-naphthylacetamide (DA992, naftipramide, *XLI*)[340]. By the oral and intraperitoneal routes, this compound possesses



(*XL*)



(*XLI*)

activity similar to phenylbutazone in rat-foot oedema induced by kaolin, dextran, 5-hydroxytryptamine, formalin or carrageenan, in formalin peritonitis, and in chronic tests using the agar granuloma and croton oil granuloma-pouch. It has antipyretic and analgesic activity similar to phenylbutazone. Blood levels of 2–4 mg/100 ml. are attained in man in the first 4 hours after ingestion of 400 mg, 50 per cent of the drug being excreted unchanged.

Anti-inflammatory properties have also been claimed for 2-phenyl-2-alkylthioethylamines[341], 2-aryl-2-amino-alkylmercaptoethanoic acids and derivatives[342,343] and 4-substituted phenylacetic and 2-phenylpropionic acids[344].

4-Isobutylphenylacetic acid (ibufenac, Dytransin), p-Me_2-CH·CH_2·C_6H_4·CH_2·CO·OH, possesses anti-inflammatory and analgesic activities 2–4 times those of acetylsalicylic acid measured by an ultra-violet erythema test and by the method of Randall and Sellito, and antipyretic properties 4 times those of acetylsalicylic acid. In a dose of 960 mg it is effective in delaying the development of thurfyl nicotinate erythema in man[345].

Chalmers[346], in controlled trials, found the drug to be effective clinically in doses of 15, 30 or 60 grains daily in the symptomatic treatment of rheumatoid arthritis. He concluded that, although not superior to acetylsalicylic acid, it appears to provide comparable symptomatic relief without comparable side-effects. Thompson, Stephenson and Percy[347] found ibufenac to be superior to calcium acetylsalicylate in terms of relief of symptoms and improvement in grip strength and that in long-term treatment the incidence of dyspepsia is reduced. Hyperuricaemia was, however, noted in some patients; the authors suggest that the occurrence of jaundice in two patients imposes a limitation on the unrestricted use of this drug. These workers found, like Tudhope[348], that faecal blood loss is slight. Hart and Boardman[349] claim that ibufenac is an effective substitute for acetylsalicylic acid and is well tolerated by patients with dyspepsia caused by salicylates.

The effect of a number of substituted arylalkanoic acids on vascular permeability in the mouse peritoneum was studied by Northover[208].

The anti-erythemic activity of a number of substituted aryloxyalkanoic acids selected from a series of mono- and di-substituted aryloxy-acetic, -propionic and higher acids[350] is described by Adams[351]. Four of these, α-4-s-butylphenoxypropionic acid, α-4-phenylphenoxypropionic acid, α-4-carboxyphenoxypropionic acid and α-4-hydroxyphenoxypropionic acid were later examined by Northover and Subramanian[121]. Although salicylate antagonizes the inflammation produced in the mouse-paw by histamine and formalin, the four aryloxypropionates are effective only against formalin oedema. The ethyl ester of α-(4-phenylphenoxy)propionic acid, which is 2–8 times more active than acetylsalicylic acid in ultra-violet erythema in the guinea-pig, later proved to be inactive clinically in rheumatoid arthritis in a dose of 1·8 g/day[11].

Various other aryloxyalkanoic acids given subcutaneously reduce vascular permeability in the mouse[175,352]. Anti-inflammatory activity has been claimed for two indanoxybutyric acids[353]. *o*-Ethoxy-substituted phenoxyethylamines, in oral doses of 25 mg/kg, are claimed to inhibit ultra-violet erythema in the guinea-pig[354].

Substituted Oxadiazoles

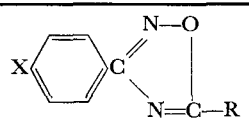
A series of a hundred 1,2,4-oxadiazole derivatives containing basic chains at C_5 and mainly aryl groups at C_3 were synthesized and examined for anti-tussive, antispasmodic and anti-inflammatory activity[355,356]. Six of these compounds were examined in more detail for antispasmodic, local anaesthetic, analgesic, anti-inflammatory and antitussive properties[357]. The formulae and activities of these compounds are shown in *Table 2.5.* Anti-inflammatory activity was determined in the rat, yeast being used to produce oedema. Three compounds with antitussive activity also exhibit analgesic and anti-inflammatory properties. It was therefore suggested that these substances, as they possess anti-inflammatory activity, might represent a novel approach to the treatment of cough. Further details of the pharmacology of one of these compounds 5-β-diethylaminoethyl-3-phenyl-1,2,4-oxadiazole (oxolamine, Perebron, AF438) have been reported[358,359]. It was also suggested that, as it has anti-inflammatory activity, oxolamine should be effective in suppressing cough associated with inflammatory changes in the bronchial tree[359], and some clinical reports seem to confirm this theory[360,361]. De Gregario[361] also claims to have demonstrated an analgesic and anti-inflammatory effect of the drug in doses of 2 g daily in the rheumatic and dental fields.

From the urine of mice, rats, dogs and men treated with oxolamine, diethylamine, small amounts of unchanged drug and a derivative thought to be 5-hydroxyethyl-3-phenyl-1,2,4-oxadiazole were isolated[362].

The pharmacological activities of two more compounds were later described. 5-β-Diethylaminoethyl-3-α-phenylpropyl-1,2,4-oxadiazole citrate (AF634) seems to have an activity similar to that of oxolamine[363], but 5-diethylaminoethyl-3-*p*-methoxyphenyl-1,2,4-oxadiazole (AF594, mexolamine) seems, in the main, to possess analgesic and anti-inflammatory activities[364].

The 1,3,4-oxadiazole derivatives of salicylic acid have been mentioned earlier (p. 80).

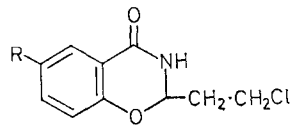
Table 2.5. Structures and pharmacological activities of some 1,2,4-oxadiazoles. (*After* Silvestrini and Pozzatti[357])

Compound			*Antispasmodic activity (in vitro)*	*Local anaesthetic activity*	*Analgesic and anti-inflammatory activity*	*Anti-inflammatory activity*	*Central anti-tussive activity (a)*	*Antitussive activity (b)*
	X	R						
AF 521	H	$CH_2 \cdot NEt_2$	++	++++−	+++++	++++−	+	++
AF 511	Cl	$CH_2 \cdot NEt_2$	−	−	−	−	−	−
AF 438	H	$CH_2 \cdot CH_2 \cdot NEt_2$	++++	+	+++	+++	++	++++++
AF 520	H	$CH_2 \cdot CH_2 \cdot NMe_2$	−	−	++−	++++−	+++	++++
AF 518	Cl	$CH_2 \cdot CH_2 \cdot CH_2 \cdot N$ 	++++++	+−	−	++++−	−	−
AF 462	H	$CH_2 \cdot CH_2 \cdot CH_2 \cdot N$ 	++++	++	−	+++++−	?	−

(*a*) Electrical stimulation of superior laryngeal nerve in the cat.
(*b*) Acrolein aerosol stimulation in the guinea-pig.

Substituted Dihydrobenzoxazines

Kadatz[365] first described the anti-inflammatory and other pharmacological properties of a benzoxazine derivative, 2-(β-chloroethyl)-2,3-dihydro-4-oxobenz-1,3-oxazine (Ap67, chlorthenoxazin, *XLII*, R = H). The compound possesses anti-inflammatory, analgesic and antipyretic properties



(*XLII*)

when given parenterally. It is more active than acetylsalicylic acid in suppressing oedema due to dextran, formalin and egg-white in the rat-foot, and about as active in reducing ultra-violet erythema in guinea-pigs. In our experience this compound, in an oral dose of 160 mg/kg, does not delay ultra-violet erythema, estimated 2·5 and 5 hours after oral dosing. Hillebrecht[366] found that the compound, when given by the intraperitoneal route, suppresses kaolin, egg-white and 5-hydroxytryptamine oedema, and Schneider and Tronnier[367] found it to have some effect on experimental ultra-violet erythema in man. Moritz[368] found chlorthenoxazin to be a good antipyretic and analgesic drug, but patients with rheumatic conditions did not always respond well to doses of 2 g daily. Higher doses were avoided because prolonged treatment with 3 g or more daily had previously caused gastric disturbance in some patients. Kendall[369] and Wilson[370] found the drug to be a useful analgesic in osteoarthritis. Bullen[371] noted no gastro-intestinal bleeding in patients receiving 1·8 g daily for 5 days. The drug is well absorbed in the rat and in man[372].

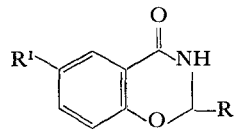
Analogues of chlorthenoxazin have been tested by several investigators[92,373,374]. One of these studies[373] (*Table 2.6*), showed 6-amino-2-(β-chloroethyl)-2,3-dihydro-4-oxobenz-1,3-oxazine (A350, ICI 350,*XLII*, R = NH_2) to be the most active. This compound has anti-inflammatory, antipyretic and analgesic properties without significant effects on the cardiovascular system[92,374–376]. The drug has been used in tablets containing 240 mg, together with 80 mg of amidopyrine (Dereuma) in the treatment of rheumatic disease[377,378] but the effects were less consistent in osteoarthritis than in those involving inflammation and swelling[377].

Some pyridylethylbenzoxazinediones were examined by Shapiro, Rose and Freedman[379], who concluded that some of these compounds have anti-inflammatory activity superior to that of phenylbutazone, but are more toxic.

Cinchophen

Cinchophen (2-phenylquinoline-4-carboxylic acid, Atophan, *XLIII*) has been used in the treatment of gout and arthritic conditions, but fell into disrepute as it caused toxic hepatic cirrhosis with a high mortality in sensitive individuals. It must nevertheless be mentioned as it represents another class of chemical compound which possesses anti-inflammatory activity.

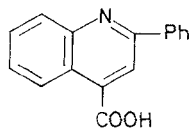
Table 2.6. Activities of some substituted dihydrobenzoxazines in inhibiting various types of oedema in the rat-foot



R^1	R	*Dose* mg/kg *oral*	% *Inhibition* (±*s.d.*) *of oedema induced by:*			*Oral* LD50 mg/kg (*with confidence limits*), *rat*
			Carrageenan	*Dextran*	*Formalin*	
H	$CH_2 \cdot CH_2Cl$	200	37·0 (±3·2)	22·9 (±4·8)	12·1 (±3·6)	>2,000
NH_2	$CH_2 \cdot CH_2Cl$	195	54·9 (±1·9)	38·8 (±4·2)	27·5 (±4·2)	1,958 (1,847–2,024)
H	Et	131	33·0 (±2·8)	24·2 (±4·7)	15·6 (±3·9)	1,310 (1,156–1,573)
NH_2	Et	189	41·5 (±2·2)	35·3 (±5·1)	18·1 (±3·4)	1,890 (1,756–1,981)
H	Me	102	25·8 (±2·4)	22·3 (±4·8)	7·5 (±3·1)	1,025 (851–1,270)
NH_2	Me	141	35·8 (±3·1)	32·6 (±5·9)	12·3 (±3·9)	1,415 (1,282–1,593)
H	$CHMe_2$	185	20·5 (±2·9)	12·7 (±6·4)	6·4 (±2·1)	1,850 (1,781–2,021)
NH_2	$CHMe_2$	200	26·4 (±2·5)	20·9 (±5·8)	16·6 (±3·9)	>2,000
Phenylbutazone		128	48·2 (±3·1)	29·9 (±4·7)	28·6 (±5·4)	1,280 (1,156–1,325)

(*From* Arrigoni-Martelli, Garzia and Ferrari[373], *by courtesy of* Journal of Pharmacy and Pharmacology, London)

Since the drug was comprehensively reviewed by Heuper in 1948[380], only recent studies dealing with certain aspects of its anti-inflammatory activity will be discussed. In subcutaneous doses of 500 mg/kg it reduces formalin oedema of the rat-foot[177] and, in oral doses of 375 mg/kg, it inhibits yeast and formalin but not 5-hydroxytryptamine oedema of the mouse-foot[118]. When given in doses of 75 and 200 mg/kg before, and 8 hours after, tuberculin, it



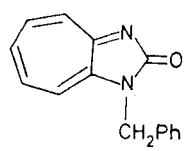
(*XLIII*)

does not reduce tuberculin sensitivity in the guinea-pig[136]. In the modified mouse 'squirming' test of Whittle, it shows both analgesic and anti-inflammatory effects in oral doses similar to those in which acetylsalicylic acid is also effective[157]. An oral dose of 200 mg/kg of cinchophen inhibits ultra-violet erythema in the guinea-pig and its derivative, oxycinchophen (3-hydroxycinchophen, HPC) is effective at 100 mg/kg[66].

Oxycinchophen is effective in various rheumatic conditions[381–385] but is no longer used because of side-effects[386,387]. A number of cinchoninic acid derivatives are alleged to possess antipyretic, analgesic and anti-inflammatory properties[388]. The clinical use of oxycinchophen in collagen diseases was based on the finding that, in rats, the compound decreases adrenal ascorbic acid[389], and has other actions suggesting that it might be effective in diseases which respond to ACTH[381]. In patients under treatment with oxycinchophen there is, however, no increased excretion of 17-ketosteroids[383,390].

RCH-314

RCH-314 (1-benzylcycloheptimidazol-2(1*H*)-one, *XLIV*), containing a seven-membered ring, possesses analgesic, anti-inflammatory and antipyretic properties[391,392]. Its analgesic activity is 2·4 and 2·6 times that of amidopyrine when given subcutaneously and orally, respectively. These



(*XLIV*)

effects are not considered to be of the morphine type because of the lack of antagonsim to nalorphine and because there is no respiratory inhibition. When given intraperitoneally it is more active than amidopyrine, phenylbutazone and acetylsalicylic acid in suppressing dextran oedema of the rat-paw, but the effect on formalin oedema, like that of the other compounds, is less marked. In hyperthermic rats intraperitoneal RCH-314 is a more potent antipyretic than either amidopyrine or phenylbutazone.

Gold and Antimalarial Compounds

There are two classes of drugs, gold compounds and antimalarials which, whilst they have been shown to possess certain experimental anti-inflammatory activities, seem, on the basis of their clinical effects, to have a delayed action against rheumatoid arthritis. Their effect appears to be more selective than that of the salicylates and other drugs previously discussed. Indeed, in view of their favourable influence on the sheep-cell agglutination titre (SCAT), they may even modify the disease process itself. Control of the disease, however, is usually incomplete and their ultimate effect is doubtful[393].

Gold compounds

The role of gold salts in the treatment of rheumatoid arthritis has been comprehensively discussed in a number of recent reviews[394–399]. The chemistry, pharmacology, metabolism and pharmaceutical presentation of therapeutic gold compounds were reviewed by Nineham[400]. Despite the fact that the value of gold in rheumatoid arthritis was first reported by Lande in 1927[401], opinions on the effectiveness of chrysotherapy have not been universally accepted. The results of the Empire Rheumatism Council's Gold Trial in 1961, a double-blind controlled multicentre study lasting 30 months, provided the most comprehensive information on the value of gold (sodium aurothiomalate, Myocrisin) yet reported[402]. Patients in the treated group were given 20 weekly intramuscular injections of 50 mg of sodium aurothiomalate. From the third month of therapy the treated patients improved to a greater degree as assessed by functional capacity, fitness, joint involvement, grip strength, SCAT, haemoglobin and erythrocyte sedimentation rate. This improvement was still present at 18 months, though reduced in degree, but at 30 months most of the improvement had disappeared. Radiological assessment of joints revealed no difference between groups. Gold toxicity usually develops during the period of injections; lesions of the skin and mucous membranes occur, and sometimes haematological complications[393].

Gold compounds also reduced an infective arthritis in mice[403] and rats[404,405], and an arthritis in rats produced by an exudate from Murphy rat lymphosarcoma[406]. Some of these effects may be the result of a direct action on infecting organisms[407]. Nevertheless, Newbould demonstrated that sodium aurothiomalate possesses moderate anti-inflammatory effects in adjuvant arthritis, a non-infective arthritis, though this was only achieved with the high intramuscular dose of 25 mg/kg/day[148].

Sodium aurothiomalate in a dose of 4 mg weekly reduces permeability of the rabbit synovial membrane[150]. The same compound, given intramuscularly in a daily dose equivalent to 0·75 mg of elemental gold, reduced the cotton-pellet granuloma in rats, an effect thought to be due to an increased turnover of adrenocorticosteroids[408].

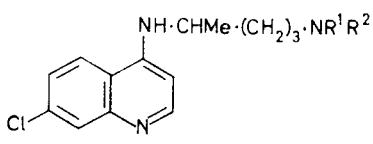
Sodium aurothiomalate, in an intramuscular dose of 25 mg/kg, reduces the leucocytic infiltration but not the exudate formation produced by the implantation of plastic sponge in rats[47]. Lawrence used sodium aurothiomalate containing ^{198}Au in patients with rheumatoid arthritis, and noted that painful joints gave counts 2·5 times higher than symptomless ones and that

fluid and fibrin clot from a popliteal bursa showed a gold concentration 67 times higher than that in the plasma[409]. He suggested that gold compounds may have a local action on inflamed tissues by inhibiting the enzymes concerned with the inflammatory process.

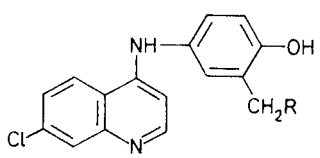
Antimalarials

In 1951, Page[410] reported that mepacrine (quinacrine) was of value in lupus erythematosus and that, in one patient with a polyarthritis of rheumatoid type, there was a simultaneous improvement of skin lesions and arthritis.

At about the same time patients with rheumatoid arthritis were found to respond to primaquine and three chemically related 8-aminoquinolines[411]. Freedman and Bach[412] reported further results with mepacrine in rheumatoid arthritis, but later Freedman[413] indicated the dangers of the drug in doses of 200 mg/day. After preliminary results with other antimalarials, which showed that proguanil was of doubtful value, Freedman[414] emphasized the benefits of chloroquine (Avloclor, Aralen, Resochin, *XLV*, $R^1 = R^2 = Et$) in doses of 200 mg or 300 mg daily in rheumatoid arthritis. Later, in a double-blind trial lasting one year, he found that patients receiving 400 mg of chloroquine sulphate and 40 grains of acetylsalicylic acid daily showed a substantial improvement over those receiving only the acetylsalicyclic acid and placebo tablets; the drug proved to be safe at this dosage[415]. Further trials[416], including a 4 year study of continuous therapy[417], confirmed the value of the drug. Many publications have subsequently appeared, one of the most interesting of which is by Popert, Meijers, Sharp and Bier[418], who concluded, from a double-blind trial, that chloroquine may be of some value as an adjunct to the conservative treatment of arthritis. They found a significant improvement in terms of clinical and laboratory characteristics and a significant decrease in SCAT, but radiological changes in the joints were not influenced.



(*XLV*)



(*XLVI*)

Several 4-aminoquinolines have now been tested and hydroxychloroquine sulphate (Plaquenil *XLV*, $R^1=Et$, $R^2=CH_2 \cdot CH_2 \cdot OH$), a closely related derivative of chloroquine, is known to possess antirheumatic properties similar to those of chloroquine[419–423]. Amodiaquine (Camoquin, *XLVI*, $R=NEt_2$) also possesses activity but seems to be too toxic for routine use[420,424,425]. However, the closely-related amopyroquin (Propoquin, *XLVI*, R=pyrrolidino) appears to possess desirable clinical activity without serious toxicity[426–428]. Drugs of the 4-aminoquinoline type in most frequent use are chloroquine phosphate, 250–500 mg daily, chloroquine sulphate, 200–400 mg daily, and hydroxychloroquine sulphate, 400–600 mg daily[393]. A striking feature of this therapy is that beneficial effects are often not apparent until

the third month of treatment. Side effects include gastro-intestinal symptoms, skin rashes, depigmentation of the hair and two more serious and apparently separate ocular complications, corneal opacity and retinal damage; the latter occurs only after long-term treatment and may be permanent[429,430].

In view of the long latent period required for chloroquine to become effective in rheumatoid arthritis, and the fact that it decreases SCAT, it seems unlikely that its mode of action is similar to that of salicylates and similarly acting compounds. It has been tested in a number of conventional and other anti-inflammatory tests, often with somewhat conflicting results.

Sharp[150] investigated the effect of a number of antirheumatic drugs on synovial membrane permeability in the rabbit. He found that chloroquine phosphate, given in a daily dose of 8 mg/kg intraperitoneally for 14 days, significantly reduces membrane permeability but commented that the relationship between these observations and the arthritic process is uncertain. Floersheim[136] tested a large number of compounds on the tuberculin reaction in guinea-pigs after sensitization with BCG vaccine. Sodium salicylate was inactive, phenylbutazone had only a slight effect and prednisolone, even in a dose of 30 mg/kg, had only a moderate effect; but chloroquine phosphate in a dose of 45 mg/kg decreased both skin swelling and erythema. However, Houba and Adam[431] claim that chloroquine phosphate does not influence the reaction when given over differing periods of time in doses of 10 mg/kg intramuscularly, although there is some evidence of inhibition in one of their tests. Pomeranz, Smith and Malak[432] also found no inhibition when chloroquine dihydrochloride was given in doses of 10 mg/kg and 40 mg/kg intramuscularly.

In rat-foot oedema tests, chloroquine sulphate in intraperitoneal doses of 10 mg/kg twice daily two days before and immediately prior to a formalin injection was found to be ineffective[95], whereas other workers have found chloroquine inhibitory in oral doses of 50 mg/kg[433]. In these latter tests mepacrine, proguanil and primaquine, in oral doses of 50 mg/kg, were also effective. Chloroquine sulphate in doses of 10 mg/kg twice daily intraperitoneally for short or long term administration does not affect the development of generalized oedema produced by intraperitoneal dextran or egg-white[434]. When administered chronically it reduces skin inflammation produced locally by chloroform, but is less effective than salicylates[434].

It is not certain that chloroquine inhibits the formation of granulation tissue. Haberland[435] found that intraperitoneal doses of 10 and 30 mg/kg of chloroquine phosphate reduce the dry granuloma weight in the rat cotton-pellet test. This *in vivo* study confirms his *in vitro* experiments in which the compounds in concentrations of 1 in 100,000 inhibited the growth of chicken fibroblasts and human and rat fibrocytes. Others showed that chloroquine sulphate in doses of 10 mg/kg intraperitoneally twice daily is ineffective in the cotton-pellet granuloma test, but reduces both the weight of the pouch wall and volume of exudate in the granuloma-pouch test[436]. In our own experience, daily oral doses of 32 and 64 mg/kg of chloroquine phosphate do not reduce the granuloma weight in the cotton-pellet test in the rat. In a silica-granuloma test in guinea-pigs, subcutaneous doses of 40 mg/kg/day of chloroquine hydrochloride, given before and during the test, reduce the weight of granuloma[432].

In the guinea-pig it was found that predosing with daily oral doses of 20, 40 and 80 mg/kg of chloroquine phosphate for 28 days does not delay ultraviolet erythema. In similar tests it was found that, after single high oral doses, chloroquine phosphate, primaquine phosphate and quinine hydrochloride are inactive, that mepacrine hydrochloride is active only at a lethal dose, and that amodiaquine hydrochloride is active only at 200 mg/kg, which is near the lethal dose[66]. An intramuscular dose of 80 mg/kg of chloroquine sulphate does not decrease the volume of exudate extracted from an implanted non-resorbable plastic sponge in rats, but the degree of leucocytic migration is significantly reduced[47].

In rat adjuvant arthritis, Newbould found that neither chloroquine nor hydroxychloroquine is effective[148], despite the fact that radioactive material was detected in the inflamed joints of adjuvant treated rats dosed with ^{14}C chloroquine phosphate[437].

The metabolism of chloroquine has been investigated by a number of workers[438–443]. Perhaps the most interesting feature is that, in patients who received large amounts, excretion continues many months or years after discontinuation. After a single intravenous injection of 5 mg/kg, the drug rapidly accumulates in ocular and other tissues, reaching peak levels in 24–48 hours. In the iris and choroid the levels remain elevated, with as much drug present after 28 days as at 48 hours, and there is only a small further decrease after 5 months[443].

A number of experiments dealing with the inhibition of formalin oedema by quinine sulphate have been reported. Theobald[177] showed that a pronounced anti-inflammatory effect is produced by quinine in doses of 50 mg/kg by either the oral or subcutaneous route. After hypophysectomy a significant though less pronounced anti-inflammatory effect is still apparent[274], whilst in adrenalectomized rats it is abolished[94]. Naumann and Stenger[444] showed that the inhibitory action of a subcutaneous dose of 50 mg/kg of quinine sulphate on formalin oedema is abolished by bilateral removal of the thyroid and parathyroid glands. After unilateral removal of the glands, slight activity persists. It thus appears from these publications that the anti-inflammatory activity of quinine may be mediated through a complex hormonal mechanism.

Catecholamines and Monoamine Oxidase Inhibitors

As mentioned previously (page 64), Spector and Willoughby[57,58] have suggested that some of the vascular phenomena occurring in tissue injury may be the result of the local destruction of adrenaline-like vasoconstrictor compounds. They measured increased capillary permeability in rats subjected to thermal injury and turpentine-induced pleurisy. The subcutaneous administration of five monoamine oxidase inhibitors, including iproniazid, markedly inhibits both types of inflammation. Iproniazid inhibition can be completely antagonized by the intravenous injection of the adrenolytic substance, dibenamine, but not by adrenalectomy. A subcutaneous dose of 0·5 mg/kg of adrenaline also reduces capillary permeability, but noradrenaline even in doses of 1 mg/kg is ineffective. From this and other evidence they conclude that in tissue injury, apart from the release of substances increasing capillary permeability, there is the release of an adrenaline-like vasoconstrictor substance which is itself destroyed by monoamine oxidase.

The greater activity of adrenaline compared with noradrenaline in oedema in the rat was demonstrated by other workers[76,445]. In a subcutaneous dose of 100 μg/kg adrenaline hydrochloride inhibits the generalized oedema produced by intraperitoneal egg-white (anaphylactoid reaction), but noradrenaline hydrochloride is only one twenty-fifth as active and isoprenaline hydrochloride inactive[445]. Parratt and West[76] found that doses of 2 mg/kg of adrenaline subcutaneously reduces foot oedema in the rat produced by a variety of agents, but that noradrenaline was only one tenth as active. They conclude that vasoconstriction cannot be the cause of this inhibition as noradrenaline is the more potent vasoconstrictor, and suggest that a metabolic action of adrenaline may be involved. That this, in fact, may be so, is suggested by the recent work of Kellett[446], who found that adrenaline is more potent than noradrenaline in inhibiting yeast-induced oedema in the rat-paw, and that this relationship can be correlated with their hyperglycaemic activity. He showed that glucose also inhibits oedema and that the inhibition by both adrenaline and glucose can be antagonized by insulin, concluding that the anti-oedema activity of adrenaline appears to be dependent on its hyperglycaemic effect.

Brown and West[447] later showed that adrenaline, when given with dextran or egg-white intradermally or subcutaneously, is considerably more active than noradrenaline in suppressing the subsequent reaction. They suggest that an effect on carbohydrate metabolism may be involved since exogenous glucose also prevents the response. However, the sympathomimetic amines in much smaller doses also inhibit inflammatory effects of bradykinin, and in this case exogenous glucose exerts only a feeble inhibitory effect. From these and other experiments they conclude that adrenaline, and possibly noradrenaline, may act as local anti-inflammatory hormones in the tissues.

Thus catecholamines may influence the inflammatory reaction through two mechanisms. On the one hand the hyperglycaemic action of large doses of exogenous adrenaline may exert an anti-inflammatory effect, whilst small quantities, such as may be liberated *in situ*, may act by direct vasoconstriction.

The situation in the mouse may be somewhat different since in formalin oedema in this species subcutaneous doses of 0·4 mg/kg of noradrenaline and isoprenaline are highly active, but doses of 4 mg/kg of phenylephrine and 20 mg/kg of amphetamine are necessary for significant inhibition[176]. Moreover, capillary permeability in the peritoneal vessels of the mouse is reduced by intraperitoneal concentrations of noradrenaline, isoprenaline, adrenaline and phenylephrine, in that order of decreasing activity[175].

Mention should be made at this juncture of the role of adrenolytic drugs as inhibitors of inflammation. Spector and Willoughby found that dibenamine hydrochloride antagonizes the anti-inflammatory effect of iproniazid, but other workers have shown that adrenolytic drugs themselves reduce capillary permeability[175,448], and that dibenamine hydrochloride suppresses the tuberculin reaction in the guinea-pig[136].

In view of the theories of Spector and Willoughby regarding the role of monoamine oxidase inhibitors in preventing the destruction of an adrenaline-like substance in tissue injury, it is not surprising that other workers have also found that these drugs inhibit inflammation. Thus iproniazid phosphate and

Table 2.7. The effects of intravenous doses of derivatives of phenothiazine on the local oedema reaction produced in rats by 5-hydroxytryptamine (5-HT), histamine, dextran, egg-white and compound 48/80

Compound	*Structure*		*Dose* mg/kg	5-HT	*Histamine*	*Dextran*	*Egg-white*	*Compound* 48/80
	R^1	R^2						
Promethazine	$-CH_2 \cdot CHMe \cdot NMe_2$	H	2 4 8	100 60 16	40 33 16	32 29 0	75 28 0	75 29 12
Chlorpromazine	$-(CH_2)_3 \cdot NMe_2$	Cl	0·5 1 2 4	60 30 30 15	60 60 30 15	22 0 0 0	22 0 0 0	90 55 60 20
Methylpromazine	$-(CH_2)_3 \cdot NMe_2$	Me	0·5 1 2 4	92 66 34 20	42 12 8 2	25 4 8 10	29 12 12 10	21 25 4 0
4670 RP	$-(CH_2)_3 \cdot N$	Cl	0·5 1 2	83 67 17	33 17 0	29 5 2	28 15 4	68 25 17
Trimeprazine	$-CH_2 \cdot CHMe \cdot CH_2 \cdot NMe_2$	H	0·25 0·5 1	100 50 54	14 18 2	50 25 4	41 17 0	59 25 4
Methotrimeprazine	$-CH_2 \cdot CHMe \cdot CH_2 \cdot NMe_2$	O·Me	0·1 0·25 1	62 25 0	16 8 5	19 4 0	23 4 0	23 6 0

(*From* Parratt and West[76], *by courtesy of* British Journal of Pharmacology and Chemotherapy)
Responses measured in terms of the maximal values (100 per cent) found in saline-treated rats.

nialamide inhibit capillary permeability in the mouse, but isoniazid, a less potent monoamine oxidase inhibitor, is inactive[175]. Iproniazid phosphate reduces oedema of the mouse-paw induced by various agents[118]. Isoniazid does not affect ultra-violet erythema[66], but increases the tuberculin reaction in the guinea-pig[136].

Phenothiazines

In assessing the anti-inflammatory activity of phenothiazines, it should be emphasized that many of these substances possess potent anti-histamine and anti-5-hydroxytryptamine properties. They therefore have anti-inflammatory activity in those reactions produced by exogenous histamine or 5-hydroxytryptamine and in those in which either or both of these amines are liberated *in situ*, after injection of substances such as egg-white, dextran or compound 48/80 into the foot[21,449,450].

The effect of phenothiazines on these types of foot oedema were investigated by Parratt and West[76], who estimated the extent of the oedema response, not directly by volume but by a visual estimation of the degree of swelling and blueing of the feet due to leakage of previously injected dye. The results of their experiments are shown in *Tables 2.7.* and *2.8.* In this type of reaction,

Table 2.8. Approximate effectiveness of some derivatives of phenothiazine in inhibiting oedema-production[76] by 5-hydroxytryptamine (5-HT) histamine, dextran, egg-white and compound 48/80

Compound	5-HT	*Histamine*	*Dextran*	*Egg-white*	*Compound* 48/80
Promethazine	8	2	2	2	2
Chlorpromazine	16	3	20	20	3
Methylpromazine	12	10	10	10	10
4670 RP	25	10	10	10	10
Trimeprazine	25	30	20	20	20
Methotrimeprazine	100	100	100	100	100

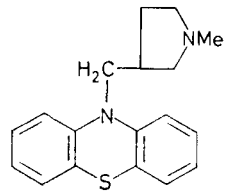
(*From* Parratt and West[76], *by courtesy of* British Journal of Pharmacology and Chemotherapy)

oedema and blueing usually progress together, though the mechanisms involved are different. It is therefore interesting to note that, with the more active phenothiazines, it is usually easier to prevent the blueing than the swelling. Methotrimeprazine is the most potent inhibitor and promethazine the weakest; these effects are probably due, in the main, to the antihistamine and anti-5-hydroxytryptamine properties of the drugs.

The effect of phenothiazines on formalin oedema, however, seems to be the result of a different pharmacological activity, since Parratt and West demonstrated that neither histamine nor 5-hydroxytryptamine plays a major role in the reaction[76]. Lish, Albert, Peters and Allen[77] concluded that there is no relationship between the antihistamine and anti-5-hydroxytryptamine actions of phenothiazines and their ability to reduce formalin oedema. This belief is further substantiated by the work of Winter, Risley and Nuss[112], who showed that cyproheptadine, a non-phenothiazine inhibitor of both

5-hydroxytryptamine and histamine[451], reduces rat-foot oedema produced by egg-white, mustard and yeast, but not that induced by formalin.

Parratt and West noted that, in doses of 1 mg/kg intravenously, methotrimeprazine temporarily inhibits formalin oedema[76]. Lish, Albert, Peters and Allen[77] found that methdilazine (*XLVII*), chlorpromazine, trimeprazine



(*XLVII*)

and promethazine reduce formalin oedema, though only the former produces statistically significant reductions at doses which are free from CNS effects. They conclude that 'the anti-inflammatory action of methdilazine and other phenothiazines is due to some other property of the individual molecule, than has been disclosed in the present study'. In other experiments in rats, promethazine hydrochloride and chlorpromazine hydrochloride in intraperitoneal doses of 10 mg/kg at 6 hours and immediately before the formalin, does not markedly reduce oedema[95]. In mice, chlorpromazine hydrochloride in a dose of 5 mg/kg intraperitoneally inhibits oedema produced by 5-hydroxytryptamine, formalin, silver nitrate and yeast[118].

Lish and McKinney[452] investigated the effects of methdilazine in a variety of anti-inflammatory tests. In the guinea-pig anaphylactic 'arthritis' test methdilazine in subcutaneous doses of 12 mg/kg produces an inhibition similar to that of 300 mg/kg of acetylsalicyclic acid. The shape of the time response curves suggests that the effect of methdilazine is due to both a non-antihistamine and antihistamine action. When administered subcutaneously methdilazine is 10–20 times as potent as acetylsalicylic acid or phenylbutazone in reducing the size of the bradykinin-induced weal in rabbits. The authors conclude that 'methdilazine opposed excessive permeability of the minute vessels by its antihistamine, antiserotonin and antibradykinin actions, plus an anti-inflammatory action not related to any of these'. The effects of methdilazine on rat-foot oedema produced by seven inflammatory agents has been reported by Winter[109].

Wilhelmi[453] found that a number of phenothiazines, administered intraperitoneally or applied locally, reduce the swelling in formalin oedema of the rat-paw: they are not, however, effective in ultra-violet erythema in the guinea-pig. In the light of the observations of Driessens[454], that promethazine in concentrations of 10–50 mg/100 ml. inhibits the growth of fibroblasts from chicken embryonic heart, he also examined the effects of phenothiazines on the regeneration of damaged tissue in salamanders and planaria. The phenothiazines markedly inhibit regeneration in both species, an effect unrelated to either the antihistamine or anti-oedema activities of the drugs.

In the guinea-pig, promethazine hydrochloride in an oral dose of 200 mg/kg prevents ultra-violet erythema, although three other non-phenothiazine

antihistamines are inactive[66]. Other workers[452] found that, although promethazine and trimeprazine do not produce even 50 per cent inhibition of the erythemic response, methdilazine inhibits at dose levels similar to those at which acetylsalicylic acid is effective.

Floersheim[136] was able to reduce the tuberculin skin reaction in the guinea-pig by the administration of doses of 10 and 30 mg/kg of promethazine and chlorpromazine, the former being the more active. Chlorpromazine hydrochloride, in an intraperitoneal dosage of 25 mg/day for seven days, also significantly reduces the permeability of the rabbit's synovial membrane[150].

Analogues of chlorpromazine in which the amino moiety is rendered less flexible by incorporation as part of a piperidine ring system, have been examined in motor activity tests and also in the guinea-pig anaphylactic 'arthritis' test. There is a retention of depressor effects on motor activity as shown by chlorpromazine but, in addition, some of the compounds also exhibit anti-inflammatory activity[455].

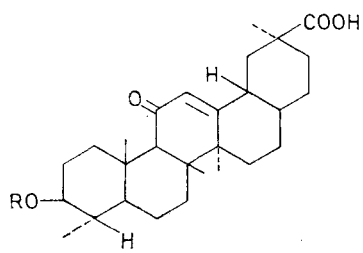
In examining the early inflammatory reaction after the implantation of pellets of plastic sponge in the rat, Saxena[47] measured exudate formation and leucocytic infiltration. The cellular infiltration was significantly decreased by the CNS depressants morphine, pentobarbitone, meprobamate and chlorpromazine hydrochloride; promethazine hydrochloride is ineffective. The extent to which this may be a direct metabolic effect of the drugs is not clear since the phagocytic activity of circulating leucocytes is decreased by chlorpromazine[456].

Chlorpromazine was found to have no marked effect in patients with rheumatic diseases associated with pain and inflammation, but it was an effective substitute in patients who had been receiving long term corticosteroid treatment for rheumatoid arthritis and from whom the drug was being withdrawn[457].

This accumulated evidence suggests that various phenothiazines can exert an anti-inflammatory effect which is unrelated to their antihistamine and anti-5-hydroxytryptamine properties.

Glycyrrhetinic Acid

The main water soluble constituent of liquorice is the glycoside glycyrrhizinic acid (glycyrrhizin). Hydrolysis of this acid yields the aglycone, the triterpenoid 'glycyrrhetinic acid'. The main component of crude glycyrrhetinic acid is 3β-hydroxy-11-oxo-18β-olean-12-en-30-oic acid (enoxolone, Biosone, *XLVIII*, R=H). The water soluble disodium salt of glycyrrhetinic acid



(*XLVIII*)

hydrogen succinate, (carbenoxalone sodium, Biogastrone, GAHS-Na, *XLVIII*, R=CO·$(CH_2)_2$·CO·ONa), has been used more recently.

Cornforth and Long[458] showed that, like hydrocortisone, certain fractions of liquorice suppress the tuberculin reaction in BCG-sensitized guinea-pigs. Later they suggested that this activity is not due to 'glycyrrhetinic acid', which they found to be inactive[459]. Finney and Somers[460], however, pointed out that not all fractions of 'glycyrrhetinic acid' have anti-inflammatory activity, and they considered that this could account for the conflicting results of the early clinical trials with these preparations in skin diseases. They demonstrated that certain fractions of glycyrrhetinic acid possess anti-inflammatory activity when administered parenterally, and when measured by the cotton-pellet method, a formalin oedema method, and the granuloma-pouch techniques in rats, and by the tuberculin reaction test in BCG-sensitized guinea-pigs. In a further paper they showed that their preparation of glycyrrhetinic acid, fraction 'S', possessed no glucocorticoid activity[461]. In large doses, however, it causes water retention, slight sodium retention, and increased potassium excretion.

The fractions used are sparingly soluble in body fluids, and it was thought that this limits the anti-inflammatory activity. The water soluble derivative, carbenoxalone sodium, was later synthesized and shown to possess a subcutaneous activity 0·23 times that of hydrocortisone hemisuccinate when measured by the cotton-wool pellet technique[462]. It appears in the blood 1 or 2 hours after being taken by mouth but is rapidly eliminated. Parke, Pollock and Williams[463], using tritium labelled enoxolone in rats, showed that most of the radioactivity is excreted in the bile in the form of three as yet unidentified metabolites. Glycyrrhetinic acid preparations are effective in the treatment of inflammatory skin conditions in dogs[464] and man[465–467]. The most interesting use of carbenoxalone has been in the treatment of gastric, but not duodenal, ulcer. The mode of action of the drug in this condition is unknown but it may promote healing by virtue of its local anti-inflammatory effect[468,469]. Its main side-effect is oedema.

N-(*2-Hydroxyethyl*)*palmitamide*

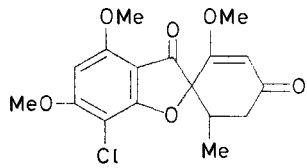
Coburn and Moore[470], and Wallis[471] suggested there is an inverse relationship between the consumption of eggs and the development of rheumatic fever in children. Experimental findings later indicated that the alcohol-soluble fraction of egg-yolk[472,473] contains a substance which reduces the inflammation of passive anaphylactic arthritis in guinea-pigs. Long and Martin[137] found that 0·006 μg/kg of a substance extracted from arachis oil depresses the allergic inflammatory response to intradermal tuberculin in BCG-infected guinea-pigs. Similar activity was found in soya bean lecithin and in egg-yolk, and Long and Martin drew attention to the possible relationship between their own findings and those of Coburn[472].

The naturally occurring anti-inflammatory agent in egg-yolk was later identified as *N*-(2-hydroxyethyl)palmitamide[474], and shown to be identical with a crystalline fraction prepared from arachis oil and soya bean lecithin which is active at 0·3 μg/kg in the guinea-pig anaphylactic arthritis test[475]. It is not active, however, in a cotton-pellet test or in a capillary permeability test in rats. When the compound is degraded, anti-allergic activity resides in

the ethanolamine but not in the palmitic acid. A number of compounds closely related to ethanolamine also possess similar activity.

Griseofulvin

Griseofulvin (*XLIX*), first isolated from *Penicillium griseofulvum* Dierckx, is effective orally in the treatment of ringworm in animals and man. The observations of Gentles[476], and Cochrane and Tullett[477], in antifungal experiments, that this antibiotic seems to possess anti-inflammatory properties led



(XLIX)

to a comprehensive investigation by D'Arcy, Howard, Muggleton and Townsend[478], who found that griseofulvin, when administered orally in a variety of experimental anti-inflammatory tests in the rat and guinea-pig, possesses activity about one-third to one-tenth that of cortisone acetate. The effects are independent of the pituitary-adrenal axis.

It has been claimed that griseofulvin has anti-inflammatory activity in arthritis, gout and similar conditions[479,480].

Aminonitriles

The ingestion of diets containing certain peas of the genus *Lathyrus* may cause widespread lesions of connective tissue, referred to collectively as lathyrism[481]. The type of lesion varies from species to species but in the rat is characterized by skeletal deformities, neurological disturbances and blood vessel rupture. The active principle in the plant has been identified as *N*-γ-L-glutamyl-β-aminopropionitrile. Similar effects can be obtained with β-aminopropionitrile itself, whilst aminoacetonitrile is even more active.

As the lesions of lathyrism are associated with connective tissue, it was of interest to study the effect of nitriles in chronic inflammation, and to compare them with corticosteroids. Mielke, Lalich and Angevine[482] using the croton oil pouch, showed that in rats treated with β-aminopropionitrile the pouch is badly formed owing to arrest of fibroblast maturation and impairment of collagen fibre formation. The tissue of the pouch has a significantly reduced content of hydroxyproline. Hurley, Storey and Ham[483] compared the anti-inflammatory effects of aminoacetonitrile and cortisone on the healing of turpentine-induced abscesses in the rat. Both drugs were given subcutaneously, the nitrile in a dose of 10 or 20 mg daily, and cortisone 5 mg daily. Inhibition of maturation of fibroblasts and delayed deposition of collagen were found in rats receiving the nitrile. Cortisone also causes reduced collagen formation but, unlike the nitrile, inhibits the proliferation of fibroblasts rather than their maturation. Studies of connective tissue formation in synthetic polymer sponge[484,485] and around subcutaneous injections of carrageenan[485] confirm the anti-inflammatory effect exhibited by the nitriles in other systems.

Levene[486] studied the relationship between structure and activity amongst the nitriles and related compounds. Modification of the nitrile group causes loss of activity. Substitution of the amino group usually destroys activity but methyleneaminoacetonitrile is active. Cyanoacetyhydrazide retains about one third of the activity of the aminoacetonitrile. Activity is also present in semicarbazide, hydrazine and several hydrazides, including isoniazid. The inhibitory effect of aminoacetonitrile, iproniazid and semicarbazide on granulation tissue formation has been confirmed[487].

The mechanism of action of the lathyrogenic nitriles is unknown. Chelation, amine oxidase inhibition[486] and adrenocortical stimulation[488] are not relevant factors.

Aminocaproic Acid

The antifibrinolytic effect of aminocaproic acid (ε-aminocaproic acid, 6-aminohexanoic acid, EACA) was discovered by Japanese workers[489,490], and the acid was subsequently shown to act mainly as a competitive inhibitor of plasminogen activation[491,492]. The pharmacology of EACA and similar compounds has been reviewed by McNicol and Douglas[493].

The relationship between fibrinolysin and inflammation has been discussed by Ungar[494] and it is, therefore, not surprising that EACA has been examined for anti-inflammatory activity. EACA, ε-acetamidocaproic acid and amidopyrine in doses of 300 mg/kg show different degrees of activity on the anaphylactoid reaction produced in rats by intraperitoneal egg-white and dextran, and on kaolin-induced rat-paw oedema: ε-acetamidocaproic acid seems to be the most active compound. In the agar pellet granuloma test there is only slight inhibition, but inhibition of histamine weals in the rat is more marked[495]. When EACA is given intraperitoneally in daily doses of 200 mg/kg to animals with the croton oil granuloma pouch, the layer of granulation tissue is about 20 per cent thinner than in controls. Histologically there is evidence of anti-inflammatory activity which may be due to a protective effect on mast cells[496].

Increased vascular permeability due to intradermal histamine in the rat, and purpura produced by subcutaneous croton oil in the mouse, are reduced by large intraperitoneal doses of EACA[497]. Anti-allergic effects of EACA have been reported in different systems[498–501], but of particular interest in the context of immunological inflammation is the finding that, on local injection, it inhibits the tuberculin reaction in man[502]. Lowney[503] noted that injection of EACA with tuberculin in humans reduces the reaction, but concluded that some if not all of the effect is due to the wealing ability of EACA. When EACA is administered in daily subcutaneous doses of 50 mg and 100 mg to rats before and during the development of adjuvant arthritis[504] the effects are not striking, though there is some evidence that the higher dose slightly affects the duration of the disease.

Amidines of Substituted Triphenylethylene and Benzylidenefluorene (Benzalfluorene)

In the search for non-steroidal substances that might possess anti-inflammatory properties like those of corticosteroids, Stucki and Thompson[104] investigated derivatives of chlorotrianisene, a substance known to possess the

oestrogenic activity shown by naturally-occurring steroids[505]. One of these, a guanyl derivative, 1,1-di-(*p*-anisyl)-2-(*p*-guanylphenyl)ethylene hydrochloride (MER-13), compound 2 in *Table 2.9*, possesses marked anti-inflammatory activity. This finding led to the investigation of other amidine hydrochlorides. Two types of test were used: oedema in the rat-foot measured 24 hours after injection of mustard and a rat cotton-pellet test. In the former test, compounds were administered either orally or subcutaneously three times daily for 3 days before the injection of irritant, and in the latter by daily subcutaneous injection. In a series of triarylethylenes (*Table 2.9*) substitution at R^1 and R^2 with methoxy, chloro, methyl or dialkylamino groups does not significantly alter activity when the compounds are given subcutaneously in the mustard test, but the hydroxy compound is inactive. In the pellet test, however, activity is unpredictable and compounds 9, 11 and 14 as well as 13 are inactive. In both tests, alteration of R has no pronounced effect when the compounds are given subcutaneously, except in the case of compounds 16 and 19. Compounds 8, 10 and 18 are orally active.

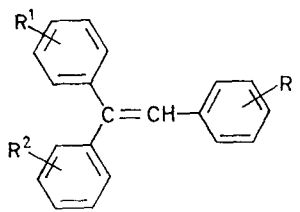
1,1,-Di-(*p*-anisyl)-2-(*p*-guanylphenyl)ethylene hydrochloride (MER-13) was examined more fully in the mustard test. The effective dose (given repeatedly) is 1·5 to 4 mg/kg, but orally it is 120 mg/kg. When administered subcutaneously, the compound is active in adrenalectomized or hypophysectomized rats. In other granuloma tests, MER-13 is active subcutaneously in a dose of 4 mg/kg/day, but, unlike cortisone acetate, inhibition is not related to dose. Moreover, in an oral dose as high as 240 mg/kg a day, no granuloma inhibition occurs. In non-inflammatory tests, MER-13 does not exhibit glucocorticoid or mineralocorticoid activity. The authors conclude that its anti-inflammatory action may bear no relation to the effect of corticoids, and that the anti-inflammatory activity is fortuitous and unrelated to the fact that the spatial configuration of certain triarylethylenes bears a resemblance to that of steroids.

Amidines of benzylidenefluorene (benzalfluorene) are also active and compounds 45 and 48 have oral activity similar to that possessed by compound 8. Compound 45, α-fluoren-9-ylidene-*p*-toluamidine hydrochloride (paranylene hydrochloride, renytoline hydrochloride, MER-27) inhibits granulation in implanted sponge pellets in daily subcutaneous doses of 25–200 mg/kg, being less active than cortisone acetate. It antagonizes mustard oedema of the rat-foot after oral doses of 10–200 mg/kg, but is not active at 200 mg/kg in adrenalectomized rats, indicating that the activity may be dependent on the adrenals[506]. In a clinical trial in 22 patients with miscellaneous arthritic disorders, the compound produced beneficial effects in 18, in doses of 100–800 mg daily[507].

Anticoagulants

Following observations which suggested that the blood clotting system plays an important role in the mechanism of inflammation, Jancsó investigated the effects of various anticoagulants given intravenously, in a variety of oedema tests in the rat-foot[508]. Heparin is ineffective but sodium polyanetholesulphonate (Liquoid), suramin and various rare earth metals such as lanthanum, cerium, neodymium, praseodymium and samarium, in the form of their inorganic salts, prove effective in reducing oedema. In most experi-

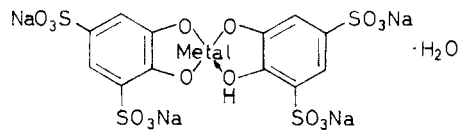
Table 2.9. The anti-inflammatory activities of some amidines of triarylethylene and benzylidenefluorene. (*After* Stucki and Thompson[104])

Compound no.	R	R^1	R^2	*Mustard irritation*				*Granuloma formation*			
				Dose mg/kg	*Activity*	*Local irritation*	*Toxicity*	*Dose* mg/kg	*Activity*	*Local irritation*	*Toxicity*
	Amidines of triarylethylenes 										
1	4-C(NH)·NH_2	4-OMe	4-OMe	25	+++	+++	++	10	−	L.I.	−
2	4-C(NH)·NH_2	4-OMe	4-OMe	25	+++	+++	++	25	++	+++	+
				Oral 30	−		−				
3	3-C(NH)·NH_2	4-OMe	4-OMe	25	++		+	25	++	L.I.	−
				Oral 30	−						
4	H	3-C(NH)·NH_2	H	25	++	++	++	25	+	L.I.	+
4*a*	H	3-C(NH)·NH_2	H	25	++	L.I.	++	25	±	L.I.	−
5	4-OMe	4-C(NH)·NH_2	4-OMe	25	++		++	25	++	L.I.	+
6	4-OMe	3-C(NH)·NH_2	4-OMe	25	++		+	25	++	L.I.	++
7	4-Cl	4-C(NH)·NH_2	H	25	+++	+++	++	25	+	+++	−
				Oral 30	−		−				
7*a*	4-Cl	4-C(NH)·NH_2	H	25	+++	+++	++	25	+	+++	−
				Oral 30	−		−				
8	4-C(NH)·NH_2	4-Cl	4-Cl	25	++	++	+	25	+	++	+
				Oral 30	++		+				
9	4-C(NH)·NH_2	2-Cl	4-Cl	25	++	+++	++	25	−	++	−
10	4-C(NH)·NH_2	4-Cl	4-OMe	25	+++	+++	+	25	+	++	−
				Oral 30	+		−				
11	4-C(NH)·NH_2	4-Cl	4-Me	25	+++	++	++	25	−	++	−
12	4-C(NH)·NH_2	4-Me	4-Me	25	+++	+++	++	25	++	+++	+
13	4-C(NH)·NH_2	4-OH	4-OH	25	−	+	++	25	−	+	−

14	4-C(NH)·NH$_2$	4-NMe$_2$	4-NMe$_2$	25	++	+++	++	25	−	+	−
				Oral 30	−		−				
15	2-C(NH)·NEt$_2$	4-OMe	4-OMe	25	Lethal			25	+	+++	+
				10	+	L.I.	++				
16	4-C(NOH)·NH$_2$	4-OMe	4-OMe	25	−	−	−	25	−	−	−
17	4-C(NH)·NH·C$_2$H$_4$·NEt$_2$	4-OMe	4-OMe	25	+++	+++	++	25	++	+++	++
				Oral 30	−		++				
18	4-C(NH)·NH·CH(CH$_2$)$_3$·NEt$_2$ (Me)	4-OMe	4-OMe	25	+++	++	++	25	++	++	+
				Oral 30	+		−				
19	4-C(NH)·NH·NH·CO (HO, OCH$_3$)	4-Me	4-Me	25	−	−	−	25	−	−	−
20	4-C(NEt)NH$_2$	4-OMe	4-OMe	25	+++	+++	++	50	++	+++	+
				Oral 10	−		−				
	Benzylidenefluorenes										
	(R^1, C=CH, R, R^2)										
45	C(NH)·NH$_2$	H	H	25	+++	+++	++	25	++	+++	+
				Oral 30	++		−				
46	CMe	C(NH)·NH$_2$	H	25	++	++	++	25	+	+	+
				Oral 30	−		++				
47	C(NH)·NH$_2$	Cl	Cl	25	++	+++	++	25	+	+	−
48	C(NH)·NH·C$_2$H$_4$·NEt$_2$	H	H	25	++	+++	++	25	++	+++	++
				Oral 30	++		++				

All compounds except compound 1 were tested as the hydrochloride salt.

ments, the didymium salt of β-acetylpropionic acid (Helodym 88) or the neodymium salt of sulphoisonicotinic acid (Thrombodyn) have been used and, although they are effective anti-oedema agents, they also prove to be toxic when given intravenously. Eventually new rare earth complexes of pyrocatechol sodium disulphonate which possess anticoagulant properties were synthesized (*L*). Such complexes of lanthanum, neodymium, praseody-



(*L*)

mium and samarium counteract inflammatory reactions induced by subplantar injection of bee venom, cobra venom, compound 48/80 or dextran in rats. The development of this type of compound as anti-inflammatory agents is the result of a singularly original line of approach.

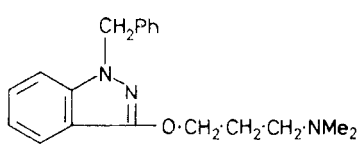
Lauenstein, Friedrich and Haberland investigated the effects of the parenteral administration of heparin, sodium polyethylene sulphonate and a number of other sulphated polysaccharides on oedema of the rat-foot produced by Zymosan[509]. Two heparin preparations were almost inactive but a heparinoid derived from fungal mycelium exhibited marked effects unaltered by adrenalectomy. These authors conclude that it is unlikely that the anti-inflammatory effects of heparinoids are dependent on anticoagulant activity.

In view of this recent work on anticoagulants, it is interesting to recall the earlier suggestion of Glazebrook and Cookson[510] that the anti-anaphylactic power of heparin and the antirheumatic effects of salicylates and of obstructive jaundice may be linked by the factor, common to all, of interference with blood coagulation. It is pertinent to record that some clinical improvement has been noted in patients with acute arthritis who were receiving sufficient dicoumarol to maintain the prothrombin index between 20 and 50[511]. Glazebrook and Wrigley[512] unsuccessfully used heparin in the treatment of rheumatic fever, but point out that a satisfactory trial of this drug cannot be made until a long acting preparation is available.

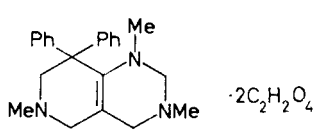
Miscellaneous Compounds

Benzindamine

1-Benzyl-3-[3-(dimethylamino)propoxy]-1*H*-indazole (AF 864, benzindamine, benzydamine, *LI*) inhibits various types of foot oedema, formalin peritonitis and granulation tissue in the rat[513].



(*LI*)



(*LII*)

It has been tested clinically as the hydrochloride and possesses anti-inflammatory activity in a variety of conditions[514–518].

Aminophylline

McKinney and Lish[519] found that aminophylline is active in oral doses of 200 or 400 mg/kg in oedema of the rat-foot produced by carrageenan, dextran and formalin, but only slightly in that induced by 5-hydroxytryptamine. On the basis of the carrageenan results, it has a potency about half that of acetylsalicylic acid. It is about one-third as active as acetysalicylic acid in delaying ultra-violet erythema in the guinea-pig, but a toxic effect in this species cannot be excluded in making this assessment. The authors speculate on the extent to which the smooth muscle relaxant or vasodilator properties of aminophylline contribute to these results, and also whether the anti-inflammatory properties contribute to the therapeutic value of the drug in bronchoconstriction.

Ro-2-5383

1,3,6-Trimethyl-8,8-diphenyl-1,2,3,4,5,6,7,8-octahydropyrido[4,3-*d*]pyrimidine oxalate (Ro-2-5383, *LII*), is four times more active than phenylbutazone and eight times more active than sodium salicylate in inhibiting yeast-induced oedema of the rat ankle, and half as active as phenylbutazone in the cotton-pellet granuloma test, sodium salicylate being inactive in the latter[179]. Its analgesic effect, as measured by sensitivity of the inflamed rat-paw to pressure, is nearly twice as great as that of sodium salicylate[50].

Ethylbutylmalonic acid di-(m-*aminoanilide*)

This compound (Gy-97), in doses of 50–200 mg/kg intraperitoneally in the rat, inhibits oedema induced by dextran, 5-hydroxytryptamine or formalin, whilst chronic treatment is also active in the croton-oil granuloma-pouch test. In rats it produces a considerable increase in blood sugar; the anti-inflammatory activity may be related to its effect on carbohydrate metabolism[520].

3-Phenylpropyl carbamate

Büch[521] investigated the activities, following intraperitoneal injection, of a series of 3-phenylpropyl carbamate derivatives on formalin, dextran and 5-hydroxytryptamine oedema in the rat-paw. All compounds showed anti-inflammatory activity and all apart from one were more active than meprobamate. Further experiments demonstrated that one of these, 3-phenylpropyl carbamate itself (phenprobamate, proformiphen, Gamaquil, MH 532), $Ph(CH_2)_3 \cdot O \cdot CO \cdot NH_2$, a central muscle relaxant with analgesic and anti-pyretic properties, possesses anti-inflammatory effects comparable to those of phenylbutazone[522]. In doses of 160 mg/kg orally, this compound does not delay the appearance of ultra-violet erythema in the guinea-pig.

Selenium compounds

Using the granuloma-pouch technique, Roberts demonstrated that certain liver fractions in doses of 200 mg/rat intraperitoneally effectively reduce exudate volume[523]. Three of six liver preparations proved to be active, as did the ash of one of these. It was later shown that the selenium content of

liver accounts for its activity and that selenium compounds, both inorganic and organic, are active at low concentrations[524]. The dose response curves for sodium selenite and for some of the organic selenium compounds are most unusual. In the case of sodium selenite, doses of selenium varying from 916 to 0·6 μg/kg were given and the most effective dose was 9·6 μg/kg. The levels both above and below this dose were less effective. A similar situation occurred for benzylselenovaleric acid. The author states that, since selenium is most effective in low concentrations (2–8 μg of Se/kg), this puts it in the trace element class, so that it must exert its effect through a regulatory mechanism.

Isoalloxazine derivatives

Büch, Molnár and Wagner-Jauregg[107] examined the effects of 6,7-dichloro-9-dimethylaminopropylisoalloxazine hydrochloride (Mol77) and 6,7 - dichloro - 9 - γ - diethylamino - β - hydroxypropylisoalloxazine sulphate (Mo242) on formalin, dextran and 5-hydroxytryptamine oedema in the rat. Intraperitoneal doses of 5 and 10 mg/kg are as active as, or more active than, 200 mg/kg of phenylbutazone in suppressing all three types of oedema. The compounds are also antipyretic in doses of 5 mg/kg. Dirscherl and Lutzmann[525] noted that Mol77 depresses the breakdown of cortisone by liver slices *in vitro*, and suggest that this may be an indication of the mechanism of the anti-inflammatory activity of flavines.

In the section on tests for anti-inflammatory activity, reasons have been given for considering that the anti-inflammatory activity of some flavines is a non-specific effect.

Metal salts

Using the granuloma-pouch technique in the rat, Sutter, Adjarian and Haskell[526] found that cuprous iodide, in a daily dose of 250 mg/kg orally, reduces the granuloma weight by 60 per cent and almost completely abolishes the exudate. This does not appear to be due to a non-specific effect as many other inorganic salts do not possess a similar anti-inflammatory activity. We found that cuprous iodide in a dose of 320 mg/kg orally, which is lethal to guinea-pigs, did not delay the development of ultra-violet erythema.

Ferrous gluconate in a daily intramuscular dose equivalent to 0·5 mg of elemental iron reduces cotton-pellet granulomata in rats; there is evidence to suggest that this effect may be due to increased turnover of adrenocorticosteroids[408].

Fisher[527] found that cobaltous chloride hexahydrate significantly inhibits the formation of exudate in the rat granuloma-pouch technique. A subcutaneous dose of the compound, equivalent to 2 mg of elemental cobalt/kg/day produces approximately the same effect as 2 mg/kg/day of subcutaneous hydrocortisone. However, when injected into adrenalectomized rats, there is no significant reduction of granuloma formation.

Citrus bioflavonoids

A group of crude and pure bioflavonoids and bioflavonoid mixtures exert a significant effect on granuloma-pouch exudate subcutaneously, but none by the oral route[528].

The anti-exudative effects vary considerably but are generally proportional to the unaltered bioflavonoid content. Thus hesperidin is active in a daily subcutaneous dose of 60 mg/kg. Studies in adrenalectomized animals suggest that the effects are independent of the pituitary adrenal axis. The possibility of a non-specific stress effect cannot be entirely ignored, but this seems unlikely since the degree of local irritation produced by the bioflavonoids shows poor correlation with their anti-inflammatory activity.

Hesperidin, neohesperidin and naringin, in a dose of 100 mg/kg intraperitoneally, reduce formalin-oedema in the mouse-foot, but are ineffective in oedema provoked by 5-hydroxytryptamine. Rutin is ineffective in both tests[176]. Other workers have found that hesperidin in large oral doses has no anti-inflammatory activity[66].

Chlordiazepoxide hydrochloride

Currie[529] reported that 7-chloro-2-methylamino-5-phenyl-3*H*-benzo-1,4 diazepine-4-oxide hydrochloride (methaminodiazepoxide hydrochloride, chlordiazepoxide hydrochloride, Librium), possesses oedema inhibitory, anti-inflammatory and analgesic effects in rats similar to those of compounds such as amidopyrine and phenylbutazone. In view of these results, the compound was subjected to clinical trial in rheumatoid arthritis and was found to have neither antirheumatic nor analgesic properties in doses up to 200 mg daily.

Other compounds which are alleged to possess anti-inflammatory activity but which will not be described in detail are: salicylanide methylcarbamate (anilamate)[530], benzoylcarbinol and derivatives[531–533], synthetic polypeptides[534], derivatives of 9-pyridylidenefluorine[535], triazinylmethylureas[536], synthetic azulenes[537–541], derivatives of $\triangle^2$-1,2,4-triazol-5-one[542], and oxyferriscorbone[543].

MODE OF ACTION

With a given series of pharmacologically active compounds, it is frequently difficult to correlate pharmacological activity with biochemical properties. In the case of a chemically heterogeneous series such as the non-steroidal anti-inflammatory compounds, this difficulty is accentuated and clearly not all anti-inflammatory compounds have the same mode of action. It does, however, seem possible that on the basis of experimental and clinical findings a number of the previously mentioned compounds or series of compounds fall into a particular pharmacological category possessing analgesic, antipyretic and anti-inflammatory activities. Thus a number of compounds mentioned in the first 9 sub-sections of the anti-inflammatory compounds section, viz. salicylates to RCH-314 inclusive, appear to be members of this group. This type of compound brings rapid relief from the acute symptoms of rheumatoid arthritis, namely, pain and tenderness, swelling and stiffness of joints, but there is no evidence that the disease process itself is affected.

It has been suggested[158] that the group of drugs known as analgesic-antipyretics can be further differentiated experimentally into those which have anti-inflammatory activity and those which do not. Only members of

the former group have proven antirheumatic activity. Thus analgesic-antipyretics which fall into the former group, all of which possess antirheumatic activity, include acetylsalicyclic acid, phenylbutazone, oxyphenbutazone, amidopyrine, flufenamic acid, indomethacin and ibufenac. Examples of the second group include paracetamol, phenacetin and 4-hydroxyisophthalic acid.

The importance of, and exact relationship between, the three pharmacological activities is not known but each seems to be an essential property which any drug must possess if it is to exhibit antirheumatic activity of this type.

Winder[544] has suggested that the antirheumatic effect of acetylsalicylic acid depends on the suppression of an early pre-inflammatory process. This process leads to stimulation of pain endings and eventually to frank inflammation. Harris[545] believes that the analgesic effects of acetylsalicylic acid can be explained entirely in terms of a peripheral anti-inflammatory action. Weiner[246] also states that the wide use of acetylsalicylic acid may be attributed to its anti-inflammatory and possibly other peripheral effects, as well as to its antipyretic action, as it is particularly effective in pain associated with fever. Yet, as Weiner has emphasized, phenylbutazone, a potent analgesic in a variety of inflammatory diseases, has little or no effect in painful non-inflammatory conditions, being, for example, of no value in headache. The work of an American team[285,286], based on the blockade of bradykinin evoked pain in the dog, has added to our knowledge of the analgesic action of a number of these compounds. They suggest that acetylsalicylic acid, sodium salicylate and phenylbutazone are peripheral analgesics which block the pain evoking chemoreceptors, thus preventing their stimulation by algesic agents.

The role of the antipyretic activity of these compounds is even less clear, but it is significant that all the known clinically effective antirheumatic drugs of this group are also antipyretics, and this property may be of fundamental importance[280]. In this context, it is of interest to note that ethyl α-(4-phenylphenoxy)propionate, an anti-inflammatory compound which does not exhibit antirheumatic activity possesses only very weak antipyretic properties[11].

With regards to the other compounds mentioned in this review, there is some justification, on the evidence available, for placing antimalarials of the chloroquine type and gold salts in a separate sub-group. This differentiation is based on the clinical properties of these drugs in rheumatoid arthritis. They possess a slower and more selective action in rheumatoid arthritis than do the analgesic, antipyretic, anti-inflammatory agents. Of particular interest is the finding that both chloroquine and gold salts affect the sheep cell agglutination titre which may indicate an effect on the disease process itself. The mode of action of these compounds is still obscure[546], though certain of their biochemical properties are discussed later. It must, however, be emphasized that the experimental anti-inflammatory action of these compounds may be unrelated to their clinical action.

In the space available it is possible to discuss only briefly certain hypotheses which have been advanced to express, in biochemical terms, the action of some of the anti-inflammatory compounds.

Protease Inhibition

The most likely mediators of the acute inflammatory reaction seem to be the kinins, such as bradykinin and kallidin. It is easy to visualize the possibility of anti-inflammatory drugs acting by inhibiting the proteolytic formation of kinins. Kallikrein is a widely distributed protease capable of forming kallidin rapidly. Plasmin is also known to be capable of liberating kinins[547], apparently by an indirect action involving the activation of kallikreinogen to kallikrein[548]. Another kinin-forming enzyme occurring in human plasma has recently been described[36]. Ungar[494] has assembled evidence to show that the plasma fibrinolytic system may play a part in liberating histamine as well as vaso-active peptides. Proteolysis is probably an essential component of the Arthus reaction[549,550] and the Shwartzman phenomenon[551].

The concept of a proteolytic mechanism in the production of inflammatory manifestations is supported by the demonstration of anti-inflammatory activity in various protease inhibitors, e.g. the trypsin inhibitors of the pancreas, soya bean, ovomucoid[552] and potato[553–555]. It is interesting to note that the inhibitor from potatoes exerts its effect even after the inflammation is well established[554], suggesting that protease action is a continuing feature of inflammation and not simply an initiating process. Various esterase inhibitors (dyflos, quinine, quinidine and chloroquine) also reduce capillary permeability induced by heat and the permeability globulins[556].

Ungar, Damgaard and Weinstein[138,494] have shown that certain well recognized anti-inflammatory drugs, salicylic acid, phenazone, amidopyrine, cinchophen and also *p*-aminophenol inhibit fibrinolysin (plasmin) in concentrations close to those which show anti-inflammatory activity *in vivo*. Salicylate may inhibit kinin formation by this indirect mechanism involving plasmin. The failure of some workers to observe any inhibition of kallikrein by salicylate *in vitro* does not conflict with this.

Lack and Ali[557] have reported that the tissue activator of plasminogen resides in the lysosomal fraction of cell cytoplasm and is inhibited by aminocaproic acid. This is noteworthy, since other workers[558–560] have postulated that the anti-inflammatory activity of cortisone may be attributable to its ability to prevent the release of lysosomal proteases by stabilizing the lysosomal membrane. Chloroquine may act similarly[560].

Antagonism of Kinin Formation and Action

We have seen above (page 62) that bradykinin may be the intermediate factor responsible for many of the manifestations of inflammation. It is natural to consider the possibility that anti-inflammatory drugs may inhibit either the formation or the action of bradykinin.

Northover and Subramanian[561] report that formation of kinin by serum and salivary kallikrein is inhibited by sodium salicylate, acetylsalicylic acid, phenylbutazone and certain other anti-inflammatory substances. The vasodepressor and vascular permeability activities of kallikrein are similarly inhibited. These findings are not, however, supported by investigations of other workers[562,563] and further studies are needed to clarify the discrepancy. It is interesting to note that hexadimethrine bromide(polybrene), which is known to inhibit kinin formation *in vitro*[564], inhibits increased capillary permeability in the xylene treated skin of mice[565].

Collier, Holgate, Schachter and Shorley[566] found that acetylsalicylic acid, phenylbutazone and amidopyrine antagonize the bronchoconstrictor action of bradykinin in guinea-pigs. Later work[209,313,567] added more anti-inflammatory drugs, including mefenamic and flufenamic acids, indomethacin and ibufenac, to the list of those active in the test. Intravenously, the minimal effective dose of acetylsalicylic acid is 2 mg/kg whilst that of phenylbutazone is 4 mg/kg and that of amidopyrine 8 mg/kg. The corresponding minimal effective doses by the oral route are 32, 16 and 16 mg/kg, respectively. The action of bradykinin on vascular permeability of the guinea-pig skin, guinea-pig ileum and rat duodenum is, however, not affected by the drugs. Acetylsalicylic acid also antagonizes the bronchoconstrictor effect of kallidin-10 in the guinea-pig but not that of substance P or angiotensin. It does not, however, inhibit the action of bradykinin in the rabbit lung[568]. Anti-inflammatory agents also antagonize bronchoconstriction due to slow reacting substances (SRS-A), which is unrelated to the kinins.

Lish and McKinney[452] studied the effects of two anti-inflammatory and two antihistaminic drugs on the diameter of weals produced in the skin of rabbits by intradermal injection of histamine and bradykinin. Acetylsalicylic acid and phenylbutazone in doses of 150 mg/kg both significantly reduce the bradykinin reaction. Chlorpheniramine, 1 mg/kg reduces the histamine reaction but 10 mg/kg fails to affect the bradykinin weal. Methdilazine, an antihistaminic drug which also has definite anti-inflammatory activity, inhibits reactions to both histamine and bradykinin in doses of 0·01 and 5 mg/kg, respectively. This work demonstrates a connection between anti-inflammatory activity and ability to inhibit bradykinin weals in rabbits. It also emphasizes the qualitative difference between the effects of histamine and bradykinin. Other workers have produced some further evidence of antikinin activity. Lecomte and Troquet[569] report that phenylbutazone inhibits the hypotension and increased vascular permeability produced by bradykinin in rabbits. A similar action is also described for dipyrone (noramidopyrinium methanesulphonate sodium, Novalgin) which antagonizes the depressive effect of bradykinin on the isolated heart of the frog, guinea-pig and rabbit[570]. Injection of bradykinin produces a nociceptive response in various species. That in guinea-pigs is not affected by acetylsalicylic acid or other anti-inflammatory agents[571], but in mice[572] and dogs[285], inhibition has been demonstrated for acetylsalicylic acid, amidopyrine and phenylbutazone.

Thus, despite the curious tissue and species specificity of the action of non-steroidal anti-inflammatory compounds on bradykinin action, it is impossible to dismiss the connection as being fortuitous.

Effects on Mucopolysaccharides

In rheumatic disease, the main pathological manifestations appear in connective tissue, the chief constituents of which are collagen, elastin and mucopolysaccharides. The last, apart from hyaluronic acid and chondroitin of cornea, are all sulphate esters[573], the sulphate groups of which are in a dynamic state with a short biological half-life. The sulphate exchange is under enzymic control and can be decreased *in vitro* and *in vivo* by corticosteroids[574–577]. Boström and Månsson[574] examined the effects of a number of salicylates on the incorporation of ^{35}S into calf costal cartilage slices *in vitro*.

They found that sodium salicylate in a concentration of 55 mg/100 ml. suppresses this to the extent of 26 per cent but that acetylsalicylic acid and benzoic acid have only slight effects. Other compounds, including *m*- and *p*-hydroxybenzoic acids, gentisic acid, salicylamide and *p*-aminosalicylic acid, all with little or no anti-inflammatory activity, are ineffective. In cotton-pellet granuloma tests, hydrocortisone 1 mg/rat and phenylbutazone 20 mg/rat given subcutaneously, decrease the uptake of ^{35}S into the granuloma. However, whilst hydrocortisone also decreases the uptake by normal connective tissue, phenylbutazone is inactive[576]. Since salicylate and phenylbutazone inhibit the transamidase which synthesizes glucosamine-6-phosphate, Bollet suggested that this might be related to their ability to decrease ^{35}S-fixation[578,579]. Gold chloride also inhibits this transamidase but acetylsalicylic acid is only slightly effective and *p*-hydroxybenzoate is inactive.

The influence of steroidal and non-steroidal antirheumatic drugs on the *in vitro* formation of cartilage and its constituent polysaccharides has been studied in tissue culture so that an examination of the biosynthesis rather than the turnover of any constituents of preformed cartilage could be made[580]. Cortisone, hydrocortisone and salicylate in high concentrations inhibit chondrogenesis and ^{35}S-incorporation, but cinchophen and phenylbutazone are inactive. The sulphation of chondroitin may be more dependent upon the supply of energy from respiration than is the biosynthesis of chondroitin.

Whitehouse and Boström[581] provide evidence that, in both cornea and cartilage, salicylic acid and some anti-inflammatory steroids reduce the uptake of extracellular sulphate ions by the tissues and also partially inhibit the incorporation of intracellular inorganic sulphate into the sulphated polysaccharides. 2,3-Dihydroxybenzoic acid is also effective, though to a lesser extent than salicylic acid; 2,5- and 2,6-dihydroxybenzoic acids are inactive. The authors suggest that these effects may be due to a common action of the drugs on processes generating ATP within the tissues. *In vivo* experiments[582] demonstrate that 100 mg/kg of sodium salicylate intraperitoneally inhibits ^{35}S incorporation into polysaccharide sulphates of rat-rib cartilage.

Whitehouse[583], and Whitehouse and Boström[584] investigated the incorporation of glucose-^{14}C, acetate-^{14}C and $^{35}SO_4$ into mucopolysaccharide sulphates, and the oxidation of glucose-^{14}C, acetate-^{14}C, pyruvate-^{14}C and octanoate-^{14}C in cartilage and cornea. The incorporation of the above substances is inhibited by salicylate, phenylbutazone and cinchophen, all of which have an immediate effect on the reaction, and by hydrocortisone and chloroquine which are effective only after a time lag of 1–2 hours. There seems to be some relationship between the inhibition of uptake by non-steroidal compounds and their anti-inflammatory activities. It is concluded that the drugs diminish anabolic reactions in connective tissue by inhibiting fundamental exergonic reactions.

The action of drugs on mucopolysaccharide synthesis cannot explain their action in acute inflammation, but it is conceivable that those drugs which affect chronic inflammation may do so by this means.

Uncoupling of Oxidative Phosphorylation

One of the many and varied biochemical effects of salicylates is the uncoupling of oxidative phosphorylation. In other words, the formation of

adenosine triphosphate, which is normally coupled to the oxidation of Krebs cycle intermediates, hydroxybutyrate and other substrates, is inhibited by concentrations of salicylate which do not affect the oxidation reactions[585–587].

Since phenylbutazone also uncouples oxidative phosphorylation, a connection between uncoupling action and anti-inflammatory activity has been suggested[68]. That the relationship is not a simple one is clear from the fact that the 'classical' uncoupler, 2,4-dinitrophenol, does not appear to possess any anti-inflammatory activity, although it has been claimed to have such activity when applied locally[588]. Whitehouse and Haslam[589], and Whitehouse[590,591] tested a wide range of anti-inflammatory compounds and found a general parallelism between their activity in this respect and their power to uncouple, as well as to inhibit phosphorylation and sulphation reactions in connective tissue. The following are potent uncouplers, reducing the P/O ratio to below 50 per cent when active in concentrations of not more than 10^{-3}M: salicylic acid, phenylbutazone, oxyphenbutazone, indomethacin, ibufenac, sodium aurothiomalate, cinchophen, glycyrrhetinic acid, mefenamic acid and flufenamic acid[589,590]. Whitehouse has used the uncoupling property to characterize antirheumatic, anti-inflammatory drugs and to distinguish them from analgesic drugs which are devoid of anti-inflammatory activity[592]. This affords biochemical support for a concept earlier made by Adams[158] on pharmacological grounds.

A further distinction can be made between rapidly acting antirheumatic agents which uncouple and slow-acting ones, such as chloroquine and the corticosteroids which, though not uncouplers, inhibit oxidative processes and may therefore achieve, by a different means, the same interference with anabolic processes essential for connective tissue function.

Whilst it seems possible that certain of the effects of anti-inflammatory compounds *in vivo* may be explained in terms of uncoupling activity (e.g. the increased oxygen consumption and hyperthermia in animals and man subjected to overdoses of acetylsalicylic acid), further work is needed to establish that lower doses of the drugs do, in fact, cause uncoupling, and that their anti-inflammatory activity is dependent on this.

Inhibition of Hyaluronidase

The relationship between hyaluronic acid, hyaluronidase and rheumatic diseases has been discussed by Smith[169], who examined the theory that the therapeutic action of salicylate in rheumatic fever might be due to inhibition of increased hyaluronidase activity, but concluded that there was little evidence to support this view. Recent work by others tends to support these conclusions[593]. Nevertheless, the subcutaneous injection of hyaluronidase into the foot of the rat leads to the development of oedema which can be suppressed by anti-inflammatory drugs[87,594]. Dewes[594] investigated the inhibition produced by a number of antirheumatic drugs administered subcutaneously and found phenylbutazone, amidopyrine, phenazone and cinchophen active, sodium salicylate less active, and acetylsalicylic acid virtually inactive. All the compounds were, however, given in high doses. Rechenberg has reviewed the actions of phenylbutazone on hyaluronidase *in vitro*, and on hyaluronidase-induced tissue permeability[273].

By means of a study of the hyaluronidase-induced intracutaneous spreading of dyes in rats, it was shown that the inhibition of spreading by salicylates, phenylbutazone and glucocorticoids is due to their ability to release catecholamines from the adrenal medulla[595–601]. This prevention of spreading is blocked by ergotamine tartrate. Chloroquine diphosphate also prevents spreading of a dye, but this effect is not prevented by ergotamine and is therefore presumably due to a different mechanism[602]. The effect of phenylbutazone analogues on hyaluronidase spreading in rats has also been examined; all show a marked inhibition in rather high doses but there seems to be little correlation with their known anti-inflammatory activities[603,604].

The available evidence does not allow a definite conclusion to be made, especially as much of the animal work has been performed with rather high doses of the drugs. It seems unlikely, however, that hyaluronidase inhibition plays a major role in clinical antirheumatic activity.

Stimulation of the Adrenal Cortex

The evidence in favour of a relationship between non-steroidal antirheumatic activity and the pituitary-adrenal system is based mainly on experiments involving salicylates and phenylbutazone. No attempt will be made to discuss these problems in detail since they have been adequately reviewed by Smith[605] and Done[606] for salicylates, and by Rechenburg[273] for phenylbutazone. Smith argued persuasively that the available experimental evidence on salicylates does not support the view that they either mimic or reinforce the actions of the natural adrenocortical hormones, and that the similar clinical effects of salicylates and these steroids in rheumatic diseases must therefore be produced by different mechanisms. Done, on the other hand, suggests that the concept cannot be prematurely dismissed and believes that the possibility that salicylate simultaneously affects the production and disposition of adrenocortical hormones deserves further consideration. He emphasizes that the antirheumatic effects of salicylates are not dependent on the maintenance of elevated circulating levels of corticoids.

More recently, a similarity has been noted between urinary electrolyte changes after large doses of salicylate in man and those following stress or hydrocortisone administration[607]. Further evidence, however, indicates that these findings may not reflect pituitary-adrenal stimulation[608]. Perfusion of the dog adrenal with salicylate does not stimulate hydrocortisone secretion[609] and there is no evidence to indicate that salicylates potentiate the thymolytic action of adrenal corticosteroids[610]. Despite the confusing evidence, it seems doubtful that phenylbutazone influences the pituitary, and any stimulation of the adrenal cortex is probably slight and non-specific[273]. There is, however, some indication that phenylbutazone *in vitro* inhibits the metabolic inactivation of adrenal steroids[273].

Of the more recently introduced antirheumatics, indomethacin is equally active in anti-inflammatory tests in intact and adrenalectomized rats[112], and there is no evidence of adrenal dependence or corticoid hormone effects for flufenamic acid at non-toxic dose levels[280].

The weight of the evidence seems to suggest that the antirheumatic action of acetylsalicylic acid and phenylbutazone is not mediated through stimulation of the pituitary-adrenal axis. If the inhibition of sulphate uptake by

cartilage slices is related to antirheumatic activity, then the effects of non-steroidal agents *in vitro*, when there can be no hormonal intervention, reinforces this view.

Chelation

Reid and his colleagues[231] put forward the theory that the anti-inflammatory action of salicylate is due to its power to form metal chelate complexes. On this basis, it would be predicted that 2,6-dihydroxybenzoic acid (γ-resorcylic acid) would be an even more potent anti-inflammatory agent. As already seen (page 77), early hopes of increased potency of this compound were not fulfilled. Nevertheless, the fact remains that many anti-inflammatory substances form coloured complexes with ferric ions in circumstances in which chemically related but inactive compounds do not[584]. Salicylate, phenylbutazone and cinchophen behave in this way. Wiesel[611] described metal chelate compounds of the anti-inflammatory glucocorticoids and showed that 8-hydroxyquinoline, well known as a chelating agent, also has anti-inflammatory activity in formalin oedema and granuloma tests in rats. Wiesel hinted that new and highly active anti-inflammatory compounds were being made on the basis of the chelation theory but these do not seem to have materialized.

Hyperglycaemia

The possible role of hyperglycaemia in relation to the anti-inflammatory activity of catecholamines has already been discussed. There is a considerable amount of additional evidence that under certain conditions hyperglycaemia produces anti-inflammatory effects.

Edlund, Löfgren and Väli[612] note that intravenous alloxan in a sub-diabetogenic dose is a potent inhibitor of weals produced by intradermal dextran. Others report that a single subcutaneous injection of insulin increases dextran oedema and that this occurs despite cortisone pretreatment[613]. In a more detailed investigation[614], alloxan-diabetic rats failed to show characteristic oedema and elevated plasma histamine levels after intravenous injection of egg-white or dextran. Pretreatment with insulin not only allowed them to respond typically, but it also made normal rats more susceptible to the reaction. It has also been shown that significantly less granulation tissue is formed around subcutaneously implanted cotton-wool pellets in alloxan-diabetic rats than in normal animals[615]. As previously mentioned, compound Gy–97 (p. 115), which inhibits rat-paw oedema induced by a variety of agents, also produces hyperglycaemia in rats. Its anti-inflammatory action is thought to be related to its effects on carbohydrate metabolism[520]. In the alloxan-diabetic rat, Kellett[616] showed that there is a significant reduction in the primary inflammatory response of adjuvant arthritis, and that the appearance of secondary lesions is delayed and their number decreased. Adamkiewicz[617] reviews the relationship between glycaemia and immune responses and concludes that hyperglycaemia inhibits anaphylactoid reactions, anaphylaxis and the tuberculin reaction. He draws attention to the similarities between the parahypersensitivity reactions (anaphylactoid reaction, and histamine and 5-hydroxytryptamine shock) and true hypersensitivity, and advances the hypothesis that hyperglycaemia inhibits, and

hypoglycaemia potentiates, the antigen-antibody reaction if this involves a carbohydrate moiety.

Thus it seems that hyperglycaemia can inhibit inflammatory reactions in which hypersensitivity or anaphylactoid-type states are involved, but not, apparently, inflammation produced compound 48/80[614] or bradykinin[447]. The inhibition of granulation tissue by hyperglycaemia also cannot be explained by the Adamkiewicz hypothesis. That hyperglycaemia can affect certain types of inflammation is not in question, but its role and its limitations have yet to be clarified.

On the basis of this evidence, it is, therefore, conceivable that some compounds which possess experimental anti-inflammatory activity, may owe this to their ability to elevate blood-glucose. This mode of action does not apply to many anti-inflammatory compounds, and at least one, acetylsalicylic acid, produces hypoglycaemia[618].

Effects on Immunological Processes

The effects of salicylates and phenylbutazone on immunological processes have been reviewed by Done[606] and von Rechenberg[273]. Details of some tests in which inflammation is the result of anaphylactic and immunological reactions are given in the section dealing with tests for anti-inflammatory activity. The effects of a number of drugs in suppressing some of these reactions, e.g. tuberculin sensitivity in the guinea-pig, have been described. Some drugs may, in fact, act by impairing the immunological stages of this process, but since many also inhibit inflammatory reactions in which these processes are not involved, it seems unlikely that this can explain the mode of action of the majority which have been reviewed here.

The effect of anti-inflammatory drugs on immunological processes, viz. antibody production and the antigen-antibody reaction, are, therefore, outside the scope of this review.

The report of the Milan Symposium, 1964 was published just before the completion of this review. It has therefore not been possible to refer in detail to all of these papers.

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3

THE PHARMACOLOGY OF HEPARIN AND HEPARINOIDS

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INTRODUCTION

HEPARINS and heparinoids constitute a group of compounds which have been shown in the past two decades to have many remarkable pharmacological properties. Their potency as pharmacological agents is usually overlooked because they do not cause the dramatic crises of classical pharmacology—respiratory and cardiovascular collapse. In fact, many of their pharmacological effects appear to be favourable to the physiologic economy.

Attention was first drawn to this group of compounds by an easily observed property of the parent substance heparin: namely, its ability to prevent the clotting of blood, commonly referred to as its anticoagulant ability. A number of workers, between 1881 and 1910, extensively studied a substance with this property, which they called antithrombin, which was found in tissues and in the blood of dogs after the injection of peptone or antigen protein. Bayliss[1] in 1916, suggested that this material, as isolated by Doyon, provides a drug for the treatment of thrombosis. The name heparin[2] was introduced by Howell and MacLean in 1918. After the demonstration early in 1937 of the efficacy of heparin in the prevention of experimental thrombosis, heparin was introduced into medicine as the first of the anticoagulant drugs, and is now used extensively for the clinical treatment of postoperative thrombosis, pulmonary embolism and coronary thrombosis. For a description of the clinical use of anticoagulants in thrombosis and the pharmacological considerations involved, the reader is referred to recent reviews in anticoagulant therapy[3–9].

The subject of the present review stems from the discoveries of A. Fischer and E. Jorpes. Fischer[10] demonstrated that heparin binds or complexes with proteins and other bases and so modifies their biological activity. As a result, heparin is able to release or activate enzymes such as lipoprotein lipase[11], to inhibit hormones such as cortisone[12] and aldosterone[13], to detoxify toxic agents[14], and to bind histamine in body cells[15]. Jorpes discovered[16] that heparin is a highly sulphated polysaccharide and that it gives a specific colour reaction with dyes; the metachromatic reaction. This resulted in (i) the association of heparin with the naturally occurring mucopolysaccharides; (ii) the preparation of other sulphated polysaccharides, heparinoids; (iii) the identification of the mast cells as heparinocytes.

Definitions

Heparin is a term which may be used in three ways, depending upon the author. It is used for any heat-stable naturally occurring anticoagulant activity; for a commercial product of varying composition with anticoagulant

and other activities; for a distinctive group of sulphated mucopolysaccharides, which can be distinguished by certain precise chemical and biological properties—formation of a crystalline barium salt with well defined chemical composition (12 per cent S, 2 per cent N, $[\alpha]_D^{20} = +55^0$); extreme solubility in water but insolubility in organic solvents; relatively low viscosity in aqueous solution; exceptional ability to combine with proteins to alter their physical and biological properties and produce metachromatic colours with basic dyes.

Heparinoids are sulphated polysaccharides, prepared semi-synthetically by sulphation of partially degraded polysaccharides or occurring naturally in plant or animal tissue. They possess the properties described for heparin mucopolysaccharide.

Mucopolysaccharides are naturally occurring carbohydrate polymers usually containing glucosamine and a uronic acid. They share, to some degree, the properties listed for heparin and heparinoids. They contain less sulphur, are much weaker as metachromatic and anticoagulant substances, and differ in solubility characteristics. Their solutions show considerable viscosity.

Heparin and heparinoids were developed because their anticoagulant properties were useful in the treatment of thrombosis; they may, however, cause haemorrhage. The corresponding biological processes are blood coagulation, thrombosis, haemorrhage. These are related, but distinctly separate biological phenomena linked to a physiological function—haemostasis. *Blood coagulation* refers to the changes which take place in blood when it is shed, resulting in the blood clot. *Thrombosis* is the development of one or more deposits inside blood vessels during life. *Haemorrhage* is the loss of blood from the circulation. *Haemostasis* is the property of the blood circulatory system whereby the circulating fluid is kept within the vessels. Heparin and heparinoids possess marked antilipaemic activity, i.e. the administration of these compounds results in the appearance in the circulation of enzymes which hydrolyse lipid attached to protein in the plasma or in a test substrate system.

Mucopolysaccharides

Mucins, mucoids, mucoprotein and mucopolysaccharide, are terms which have been used for various materials isolated from natural sources. The names first referred to the physical properties or viscosity of the materials. However, as they were subjected to chemical study, it was found that a part of the molecule was a substituted carbohydrate. It has been found that the common constituent is the amino sugar, 2-amino-2-deoxy-D-glucose or D-glucosamine. Also frequently found are the uronic acids, glycuronic or galacturonic acid, and sulphuric acid. When simple hexoses are present they are usually glucose with mannose. Jeanloz[17] has recently defined these terms more precisely, giving more meaningful equivalents. Mucus and/or mucoids are glycopeptides or polysaccharide-protein complexes. A mucopolysaccharide may be a polysaccharide-protein complex, a glycoprotein, or a polysaccharide (distinguished by containing an amino sugar). In the last case, it is a glycosaminoglycan. When in addition it contains an acid or is an acid mucopolysaccharide, it is a glycosaminoglycuronoglycan. The commonly occurring mucopolysaccharides are shown in *Table 3.1*, with their chief components. It

has been assumed that the major objective in studying mucopolysaccharides has been the removal of all protein and peptide from the preparation. Now it is realized[19] that many of the biological actions of mucopolysaccharides

Table 3.1. List of mucopolysaccharides[17, 18]

Common names	*New nomenclature (Jeanloz)*	*Chief components*	*Location*
Chondroitin sulphate A	Chondroitin 4-sulphate	*N*-acetylgalactosamine-4-sulphate; glycuronic acid	Aorta, bone, cartilage, cornea, lig. nuchae
Chondroitin sulphate C	Chondroitin 6-sulphate	*N*-acetylgalactosamine-6-sulphate; glycuronic acid	Cartilage, nucleus pulposus, tendon
Chondroitin sulphate B (β-heparin, dermotoidin sulphate)	Dermatan sulphate	*N*-acetylgalactosamine-4-sulphate; iduronic acid	Aorta, heart valves, lig. nuchae, skin, tendon
Chondroitin		*N*-acetylgalactosamineglycuronic acid	Cornea
Keratosulphate	Keratan sulphate	*N*-acetylglucosamine-6-sulphate; galactose	Bone, cornea, nucleus polposus
Heparin monosulphuric acid		Mixture of heparan sulphate, dermatan sulphate and heparin	Intestine, lung
Heparitin sulphate	Heparan sulphate	*N*-acetylglucosamine-6-sulphate; *N*-sulphato glucosamineglycuronic acid	Amyloid, aorta
Hyaluronic acid	Hyaluronic acid	*N*-acetylglucosamine-glycuronic acid	Synovial fluid, vitreous humour

depend on their relation to protein and that more is involved in this than salt linkages.

Heparin may be classified as a mucopolysaccharide on the basis that it contains glucosamine, but it has very distinctive properties, and it is better to recognize a separate class of natural compounds, the heparins or heparinoids. In this review, the term mucopolysaccharide is limited to the substances in *Table 3.1*, as distinct from the heparins and heparinoids, listed in *Table 3.2*. Sulphation of mucopolysaccharides results in heparinoids when the product resembles heparins in the chemical and biological properties included under definitions. An important biological difference is that the typical mucopolysaccharides, such as the *chondroitin sulphuric acids*, are the important structural materials of connective tissue, while the naturally

occurring heparins and heparinoids are trace substances and appear to be associated with special cells.

Heparins and Heparinoids

Heparins and heparinoids are grouped in *Table 3.2.* Some are naturally occurring compounds, whilst others are derivatives prepared from heparins or from mucopolysaccharides. Semi-synthetic compounds have been prepared by degradation of natural polysaccharides followed by sulphation with chlorsulphonic acid or methyl sulphate[20]. The common activities shown by the heparins and heparinoids—complexing with organic bases and proteins, antilipaemic activity and anticoagulant activity—are also shown by various sulphonic acid dyes and by polyphosphates. The table is completed with a list of those preparations that have been issued to provide depot preparations of heparins and heparinoids.

Table 3.2. Heparins and heparinoids

A. *Naturally occurring*

Origin and name	*Components*	*Common or commercial names*	
Beef α-heparin	6-SO_4-*N*-SO_4-glucosamine, 2- or 3-SO_4-glycuronic acid	Heparin, liquemin, thrombophob	
Pork α-heparin	6-SO_4-*N*-SO_4-glucosamine, 2- or 3-SO_4-glycuronic acid 2- or 3-SO_4-iduronic acid (?)	Heparin	
Whale-heparin	*N*-SO_4-glucosamine, *N*-acetyl-glucosamine, SO_4-glycuronic acid		(21)
Dog-heparin	?		
Clam heparin			
(*Mactrus pussula*)	?	Mactin A	
(*Artica islandica*)	?	Mactin B	
Duodenal heparinoid	Sulphated glucosamino-glycuronoglycan	Epd. D	(22)
Heparinoid intestine	Glucosaminoglycan	Ateroid	(23)
Heparinoid pancreas	Glucosaminoglycan		
Seaweed polysaccharides	?	K-carrageenin λ-carrageenin	
Heparamin	Glycuronic acid, glucosamine, sulphuric acid	Aretid	

B. (i). *Derived from heparins*

Name	*Derivation*	*Common or commercial names*	
Oxidized heparin	Heparin with H_2O_2		(24)
ψ-heparin	*N*-desulphated heparin	Heparamine	(25)
N-resulphated heparin	Chlorsulphonic acid + ψ-heparin		
Resulphated heparin	Chlorsulphonic acid + hydrolysed heparin		
N-Benzyl heparamine & *N*-monomethyl-3,5-dimethyl-benzheparide amide	ψ-heparin conjugated with chlorides	Heparides	(26)
N-heparyl-*N'*-n-butyl urea	ψ-heparin + an isocyanate	Urea derivatives of heparin	(27)
N-(2,4-Dimethylbenzoyl, *N*-desulphoheparin)			

B. (ii). *Derived from mucopolysaccharides*

Name	*Derivation*	*Common or commercial names*
Sulphated mucoitin sulphuric acid ester	Chlorsulphonic acid and mucoitin HSO_4	Elheparin Eleparon (R)
Sulphated chondroitin sulphuric acid	Chlorsulphonic acid and chondroitin sulphate A or C	
Sulphated hyaluronic acid	Chlorsulphonic acid and hyaluronic acid	
N-sulphated deacetylated blood group A mucopolysaccharides of hog gastric mucosa[28]		

C. *Sulphated polysaccharides*
(common or commercial names in brackets)

Sulphuric acid esters of: cellulose, starch, snail polysaccharides (charonin sulphuric acids), glycogen, inulin, trihexosan (sulphopolyglucan, SPG), polyglucose, polylactose, dextran (dextran sulphate, dexulate, angerin), laminarin (laminarin sulphate), xylan, galaktan, levan, levoglucosan, carrageenin, alginic acid (Norgine, Paritol), pectin (Eparoid, Heparoid, Treburon, Thrombostop, G31150, Stypturon), pectinic acid (Frenemo, Mepesulphate), polygalacturonic acid-methyl ester-methyl glycoside, chitin, chitosan, gum arabic, maltotriose, maltotetrose, maltopentanose, *N*-formylchitosan (R01–8307)

D. *Other sulphated compounds*
(common or commercial names in brackets)

Sulphuric acid esters of: tri-β-hydroxyethyl-β-propane tri-carboxylic acid ammonium (ThromboHolzinger), polyethylene sulphate sodium, polyanethol (liquoid), polyvinyl alcohol (elvanol), yeast nucleic acid, lignin, saponin, tannin, oromucoid, proteins

E. *Sulphonic acid dyes* F. *Aryl polysulphonates*

G. *Polyphosphates*

Phosphorylated hesperidin, K sodium polyphosphates, polymetaphosphates.

H. *Depot preparations*

Heparin + 10 per cent polyoidone + 1 per cent procaine (Héparine-retard, Chocy); 40,000 I.U. heparin/ml. + 20 mg ephedrine HCL (Heparin retart 'Richte (R)', Depot-Thrombophob (R)); heparin + ? (Liquemin Depot (R)), 50,000 I.U./2 ml. (Heparin-Depot-Noro (R)).

With the exception of one activity (the power to prevent clotting of whole blood), the naturally occurring prototype is not remarkably different from many of the compounds which have been prepared. However, it was the ability of the material prepared by Howell from dog liver and to which he gave the name heparin, to prevent clotting of blood, that first aroused commercial interest in these substances. It was, therefore, a matter of chagrin that the first commercially produced purified heparin proved to be weaker than Howell's reported purified material. This dilemma was resolved when it was shown that dog heparin had two and a half times the potency of beef heparin in this particular activity. This difference is well established and has been shown by four different laboratories using their own assay methods. Further differences have been shown to exist in heparin from other mammalian species, including pigs, sheep and rats[29], and recently from one species of whale (*Balaenoptera physalus Linne*[30]), which has higher activity than dog heparin. These heparins also show differences in solubility. This is most marked with heparin from mast cell tumours[31]; Green and Day[32] drew attention to a 'unique heparin' on this basis, in the rat and mouse.

A number of authors have used the prefix α- to denote the commonly used commercial heparin, prepared from beef or pig tissues. Japanese workers[30] have extended this scheme by calling whale heparin, w-heparin. On this basis dog heparin becomes d-heparin. At present there is no rational basis for the different use of 'heparin' and heparinoid'. It appears to be common custom to reserve 'heparin' for material with high anticoagulant activity and 'heparinoid' for that with lower anticoagulant activity. As present-day semi-synthetic compounds have lower activity by the U.S.P. assay (it is shown later that this is not necessarily true for *in vivo* assays) they are called 'heparinoids'. The presumption is that when one is obtained with higher activity, it may be termed a 'heparin'. As shown in the later section on blood coagulation tests (tests of anticoagulant activity), these measure so many diverse parameters that relative activity does not provide a sharp dividing line. Possibly 'heparin' should have been reserved for the material to which Howell[33] originally gave the name—the anticoagulant obtained from dog liver. As this was not done, 'heparin' now denotes a commercial product of variable origin and composition. 'Heparinoid' denotes the whole class of substances listed in *Table 3.2*. Where it is necessary to insert modifiers, such as species or tissue of origin or chemical treatment, they should be inserted in the text or by the system of prefixes suggested—w for whale, d for dog, ψ for *N*-desulphated, m.c. for mast cells, and so on.

It appears that the main chemical structure of heparin as determined by degradation studies by the research groups of Wolfrom, Meyer and Stacey[34,35] is an α-D-(1→4) linked backbone structure of alternating hexosamine and uronic acid in which the hexosamine is 6-SO_4, *N*-SO_4-glucosamine and the uronic acid, 2- or 3-SO_4-glycuronic acid. The ratio of 2·5–2·6 rather than 3·0 SO_4—for residues/nitrogen atom suggests that not all the uronic acid residues contain *O*-sulphate groups. Cifonelli and Dorfman[36] have identified iduronic acid as a component in pork (?) heparin. Other workers[37] found evidence of a (1→6) linkage of uronic acid to glucosamine. Gibbons and Wolfrom[38] found all but 6 per cent of the amino groups were sulphoamino groups. Whale heparin contains both sulphoamino and acetylamino groups[21]. (*See* reference 268 and note on p. 198.)

While the secondary level of chemical structure is thus being elucidated, it is becoming evident that the special biological activities of heparin can only be explained by the tertiary level or orientation of this structure. The linkage structure is related to amylose and after desulphation and reduction of carboxyl, heparin is hydrolysed by β-amylase[34]. This suggests it may exist in a helical structure. The low reducing value indicates either a high degree of polymerization or a cyclic structure.

Different values are given for the molecular weight of heparin as estimated by different methods by different investigators. The high electrostatic charge from the high concentration of acid groups (five sulphate and two carboxyl groups/tetrasaccharide unit) interferes with many of the determinations used for polymer size and shape. Measurable viscosity is often due more to accompanying protein or polysaccharide present than to the heparin or heparinoid present. A number of authors have used molecular weights of the order of 18,000[39], 17,000[40], 16,000[24] and 19,700[41]. Other studies provide values of 8000–14,000, giving a mean of 11,900 ± 200[42]. From viscosity data[43] and from

sedimentation and light scattering studies[44], molecular weights from 7600 to 11,800 have been reported for different samples and fractions of heparin.

COMPLEXING PROPERTIES OF HEPARIN, HEPARINOIDS AND MUCOPOLYSACCHARIDES

A property shared by all these substances is the ability to form complexes with many other substances. Collagen is a complex of protein (gelatin) and chondroitin sulphuric acid A, which may be dissociated by calcium chloride solutions and recombined again to give fibres. Complexes with many proteins have been described for heparin, heparinoids, mucopolysaccharides and alkylbenzenesulphonates[45] on the basis of insolubility of the products which have well-defined electrophoretic patterns. Complexes are also formed with various organic bases (e.g. benzidine, brucine, long chain aliphatic polyamines) and with dyes. Complexing is due both to ionic forces (salt formation) and non-ionic forces (hydrogen bonding). While the reaction involves initially the acid groups of the heparinoid and the amino groups of the organic base or protein, Matthews[46] concluded from a study of the complex with $Co(NH_3)_6^{3+}$ that the binding affinities depend largely on electrostatic interactions between neighbouring charged groups on the polyion. This explains the high affinities of heparin preparations. The degree of association or dissociation of such complexes depends on the affinity of the substance for the heparinoid or mucopolysaccharide, on the pH value, and on the concentration of other ions (including inorganic salts such as sodium chloride). Dissociation of the complex can be accomplished with alkali or strong neutral salt solutions, and this is used in the preparation of these substances. The formation of complexes[47] may be demonstrated by direct observation of the formation of the compound, by the alteration in the properties of the heparinoid, or by the alteration in the properties of the base. Direct analyses have shown that the new compound lacks the inorganic cation of the original heparinoid and the inorganic anion of the original amine, so that essentially a double decomposition has occurred. Complexing also occurs with smaller molecules such as inorganic salts and simple organic bases and is particularly important for the pharmacologist when using heparin.

Organic Bases and Histamine

Benzidine, cetylpyridinium chloride (C.P.C.), cetyltrimethyl ammonium bromide (cetavlon) and other long chain amines form insoluble complexes with heparinoids and are universally used as precipitating agents for heparinoids. The solubility characteristics in salt solutions have been established by Scott[48]. Brucine[49] and choline[49a] also give crystalline complexes with heparin in the presence of aqueous alcohol and acetone. The choline complex shows no loss of cholinergic activity, indicating complete dissociation in solution.

The close association of histamine and heparin release in anaphylactic shock and in peptone shock suggests that complexes of histamine and heparin are held in biological systems. Histamine binds heparin *in vitro*, as shown by displacement tests, by dialysis, and by paper chromatography[50]. Dextran sulphate, polymannuronic acid sulphate, polysulphated xylan and the *N*-succinyl derivative of de-*N*-sulphated heparin[51] give compounds with

histamine, whereas chondroitin sulphate or the de-*N*-sulphated heparin do not. The neutralization of the anticoagulant action of heparin by histamine has been described[52]. Similarly, hypertensin may be bound to heparin *in vitro* and the hypertensin can then be liberated by compound 48/80[53].

Metachromasia of Dyes

One of the most distinctive properties of these compounds is that of metachromasia. Heparin, heparinoids and mucopolysaccharides combine with any dye which possesses free amine groups. Some dyes change colour and this change of colour is termed metachromasia. Such metachromatic dyes are: azure A, toluidine blue, Bismarck brown, brilliant cresyl blue, cresyl violet, bromcresyl blue, neutral red, basic fuchsin, pyronin, and acriflavin[54]. The change of colour is due to a shift in the light absorption band of the dye to a shorter wave length and is different from the effect of pH on the dye. The dye commonly used is toluidine blue which changes from blue to red. The light absorption of the dye at 610 mμ is decreased when heparin is added, and a new absorption band at 505 mμ is observed (see *Figure 3.1*).

The colour change is also produced by phosphomolybdate and porcelain and it may be related to polyelectrolytes. While mucopolysaccharides give the reaction, heparin and heparinoids are distinctive in that they produce the colour change in highly dilute solutions (such as 1 mg/100 ml.) and with inorganic salts present. Some heparinoids have greater metachromatic activities than heparin[55]. The colour change (but not formation of the complex) is suppressed by alcohol and by heat. It can be produced in dilute aqueous solution, on paper chromatograms and microelectrophoresis agarose slides, by thick-layer chromatography and in tissue sections. While only heparin and heparinoids give this colour when present in trace amounts, the much larger amounts of mucopolysaccharides in tissues and tissue extracts, give a positive result. The staining is localized to the ground substances of connective tissue, cartilage, and the mast cells. Important in staining are dilution, pH, staining time, solvent, dehydration, and pretreatment. Vitry[56] states 'Following the work of Lison, one would like to attribute to metachromasia a specific value for identification of sulphated polysaccharide. But the discovery of other chromotropic substances, the lack of knowledge of the intimate mechanism of metachromasia and the technical difficulties of staining makes metachromasia specificity questionable'.

Plasma Proteins

Heparinoids and mucopolysaccharides react with, and modify, many of the plasma proteins. Heparin combines with fibrinogen, globulins and albumin. As judged by electrophoresis and various types of analysis and staining, the particular plasma protein components with which heparin combines are dependent upon the concentration of protein, concentration of heparin, pH value, and salts present. This explains the somewhat contradictory statements in literature about combinations of heparin with plasma proteins. The combination may result in change of solubility of the protein and reverse protein tests[58]. Heparin can modify the murexide reaction for calcium in serum[59] by affecting the calcium-protein-heparin complex. Many heparinoids

can be precipitated, for example cellulose sulphuric acid, liquoid Roche, starch sulphuric acid, cellulose sulphuric acid, cellulose glycollic acid and chitin sulphuric acid. Precipitation depends on the concentration and amount of polysaccharide, salts present, and pH. The precipitation of fibrinogen by dextran sulphate occurs with all dextran sulphates below the isoelectric

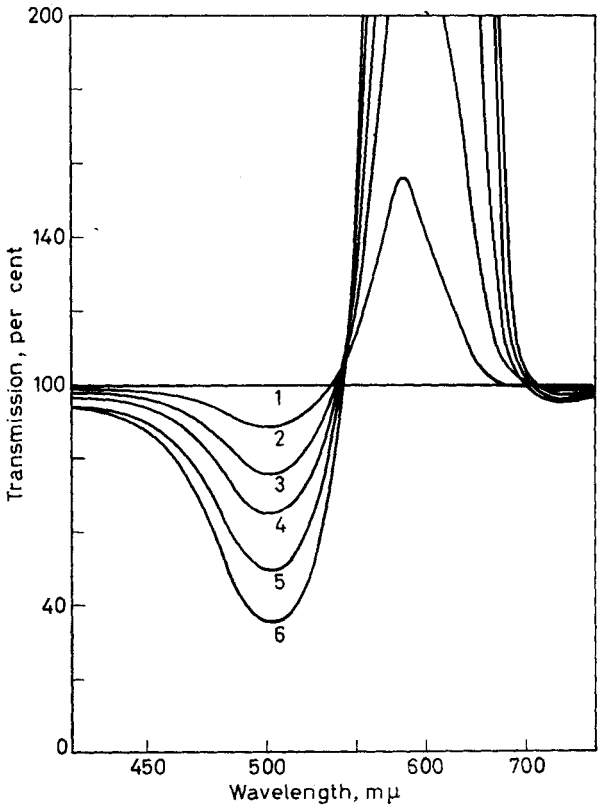


Figure 3.1. Absorption spectra for azure A solution with heparin.

Beckman DK-2 Spectrophotometer.
Azure A concentration 13 μg/ml.
1 = Reference solution or Azure A alone;
2, 3, 4, 5, 6 = 0·7, 2·1, 2·9, 5·0, 6·4 μg.
International Standard heparin/ml. added.

point[61], but above the isoelectric point this only occurs with dextran sulphates of molecular weight 129,000–458,000. Heparin appears to be specific for the precipitation of cryofibrinogen[62], and Waldenstrom's macroglobulin[63]. With globulins, heparin and a number of sulphated polysaccharides appear to precipitate specifically the serum β-lipoprotein fraction. Burstein and Samaille[64] have developed this into a useful quantitative method for the determination of β-lipoprotein, and a method for isolating these proteins for the production of specific antibodies.

Blocking Agents for Heparinoids

Many complex bases possess a sufficiently high affinity to block the activity of heparinoids in biological systems (anticoagulant, antilipemic, metachromatic activity, etc.). Examples of these are trypaflavin[65], toluidine blue[54], tetracyclines (aureomycin and tetramycin)[66], stilbamidine isothionate[67], polybrene[68], DDP (1,5-dimethyldecamethylene polymethyl bromide), hexadimethrine, histone and globin[69], and protamine[70]. Antiheparin factors are present in tissues[71]; both lipid with antiheparin properties[72] as well as basic proteins such as histones. When toluidine blue, trypaflavin, stilbamidine isothionate, and protamine sulphate were tested for neutralization of anticoagulant activity, 1 mg of mucopolysaccharide was neutralized by toluidine blue, trypaflavin, stilbamidine and protamine as follows: heparin 4·50, 2·32, 2·18 and 2·02 mg; dextran sulphate, 4·00, 1·55, 1·40 and 1·00 mg[67]. *In vivo*, protamine takes longer to neutralize paritol than heparin[73] and since larger doses of heparinoids are used, larger doses of protamine are required and may be toxic. Blocking agents for heparinoids (protamine, etc.) usually have anticoagulant action, and so blocking heparin and heparinoids requires exact equivalence. The effectiveness of these agents in counteracting heparin is also limited by other factors, such as the number of platelets present[68]. Heparin is counteracted by the substance in platelets (platelet factor 4), whereas polybrene is partially neutralized by adsorption onto the surface of the platelets. Polybrene is adsorbed by red cells, heparin is not, and platelet factor 4 is not an antipolybrene.

Action on Enzymes (including Fibrinolysin)

Heparin forms typical adducts with crystalline trypsin and crystalline chymotrypsin when tested at pH 4·0. The compounds are easily dissociated since 40 per cent of the theoretical trypsin activity and 130 per cent of theoretical heparin activity can be observed[74]. The adduct is more stable than trypsin. Heparin and heparinoids also form complexes with pepsin at pH 5·5–4·5. Direct inhibition of peptic activity is observed with α-heparin being more effective than PSC16>carrageenin>degraded carrageenin> chondroitin sulphate and β-heparin[75,76]. van Haeringen[77] concludes that with pepsin, trypsin and chymotrypsin, heparin does not cause inactivation of the enzyme but affects the substrate, particularly denatured protein. Proteolytic activity found in rabbit brain thromboplastin and in Russel viper venom, is inhibited by heparin, but heparin has no effect on 'reptilase'. Trace amounts of heparin and Elheparin appear to activate proteolysis by human serum and skin, while larger amounts inhibit it. This is also seen with the arginine esterase activity of blood[78].

The close relationship of the chief proteolytic enzyme system of plasma, the plasminogen system, to coagulation changes has led to many studies on the relationship of heparin to fibrinolysin. Heparin does not inhibit plasmin, or the fibrinolytic activity of *Aspergillus proteus*, but inhibits various fibrinolytic activators directly[80]. For antifibrinolytic activity, heparin requires a cofactor found only in plasma, and this may be the heparin cofactor itself. Heparin therefore may lack antifibrinolytic activity on a purified system due to the lack of this factor[79]. Buluk and Januszko[81] report that fibrinolysis of

bovine plasma globulins is more rapid with than without heparin, that is, an activation occurs by inhibition of antifibrinolysin. β-Heparin, chondroitin sulphate and hyaluronic acids[82] have similar activity while cellulose trisulphuric acid and alginic acid have a greater activity than heparin, and neutral polysaccharides have little effect. Ateroid only acts to normalize the fibrinolytic system when inhibition is present as from the increase in antiplasmin with lipaemia[83]. D-Xylan polysulphuric ester (SP_{54})[84] causes a decrease in antiplasmin activity of plasma and no reduction in antitrypsin activity. Halse[85] considers that the beneficial effect of heparin in cases of thrombosis is to be attributed in part to the lytic action produced, due to the activation of the precursor of fibrinolysin with heparin. Further, the inhibition of fibrinolysis in plasmas seems to be associated particularly with the β-lipoproteins and chylomicra[86]. This means then there is a significant relationship between the effects of heparinoids on the production of clearing factor and on the removal of inhibition of fibrinolysis. This is to be distinguished from any effects of heparin on fibrinolysis *in vitro*.

While heparin increases the temperature stability of trypsin and thrombin, it increases the instability of β-amylase, even though it shows no immediate inhibition. A slow reaction occurs leading to irreversible inactivation of amylase, probably due to salt formation followed by denaturation of the protein with the formation of higher complexes[87]. Heparin has no effect on the serum or plasma amylase activities[88].

Heparin, de-esterified nitrated hyaluronic acid, the related sulphonic acid dyes (e.g. chlorazol fast pink) and a non-specific inhibitor of rabbit and human serum, all inhibit various hyaluronidases from different sources but there are great differences, depending on substrate, enzyme and inhibitor. As inhibitors, cellulose trisulphuric acid and chitin disulphuric acid[89] are much stronger, while treburon is similar to heparin.

Heparin, liquoid, farma[90], congo red, trypan blue[91], and treburon[92] inhibit egg-white lysozyme. In fact, heparin and lysozyme mutually inhibit each other *in vitro* and even *in vivo*, as prolonged treatment with heparin results in reduction of salivary lysozyme values.

Pancreatic ribonuclease is markedly inhibited by heparinoids but not by mucopolysaccharides, with sulphated polyvinyl alcohol>sulphated corn amylose>sulphated cellulose>sulphated corn amylopectin>sulphated dextran, sulphated pectic acid, polyvinyl sulphonate with 21·7 per cent sulphur>sulphated nitro chitin, sulphated nitro chitosan and heparin[93]. Single injections of heparin in mice result in significant inhibition of the acid alkaline ribonuclease of the liver after the injection[94]. This probably explains the accumulation of ribonucleic acid in tissue culture cells in the presence of heparin. Heparin also inhibits rat acid phosphatase, glucuronidase[95], catalase[96], fumarase and elastase.

Possibly the most important relationship of heparin to enzymes is with liproprotein lipase, which is a mucoprotein containing mucopolysaccharide. It is remarkable in that (like the coagulation system) it is inhibited by both polycations and polyanions. This is not due to the formation of different components with enzyme and substrate, as the kinetics are the same for both inhibitions. While change in ionic concentration or pH has little effect, the inhibition must be fundamentally electrostatic. The relative activity of

various cations is such that high molecular weight compounds have greater activity than low molecular weight, e.g. dextran sulphate is more active than heparin. Highly branched compounds are much less active than other compounds. Dextran sulphate is a non-competitive inhibitor with both soluble lipoproteins and activated coconut oil, while heparin and PGS show no inhibition on soluble lipoproteins but give competitive inhibition with activated coconut oil. Korn[97] concludes that the polyanion and substrate are bound at neighbouring sites of the enzyme.

Haemolysis and Anticomplementary Activity

When a number of substances were tested for the inhibition of *in vitro* haemolysis and agglutination of red blood cells by anti-erythrocyte serum[98], the minimum active concentration in parts per million was: heparin (1:1000), liquoid (1:100,000), sulphomucoitin (0·05), treburon (0·1–1), germanin (0·1), protamine (1), neodymium salt (1), whilst β-heparin, sodium citrate, potassium oxalate, and dicumarol anticoagulants gave no inhibition. Haemolysis caused by saponin, digitonin, and sodium taurocholate[99] was not affected by heparin, β-heparin, sodium polyanetholesulphate, or treburon, whereas haemolysis with cobra and bee venom was inhibited by these substances in high dilutions. Both sets of results suggest that the effectiveness of heparin and related compounds in inhibiting haemolysis is associated with the attachment of antibody or enzyme to the cell. Heparin causes slight spontaneous haemolysis. It interferes with a number of important serological reactions. Heparin reverses the L.E. reaction in systemic lupus erythematosus. In idiopathic acquired haemolytic anaemia (IAHA), heparin causes reversal of the positive direct and indirect Coomb tests, and when used for treatment results in objective haematological improvement[100,101]. Heparin has also been successfully used for paroxysmal nocturnal haemoglobinuria.

Panasewicz[102] found that heparin prevented shock from transfusion of heterogenous blood in cats and rabbits (outdated human blood Group B). It also prevented the heterohaemolysis *in vivo* and *in vitro* but not the heteroagglutination *in vitro*, and he found this was due mainly to the inactivation of complement. With the specific blood group reactions[103], the intensity of the iso-agglutinin reaction is decreased by pure heparin, thrombophob, thrombocid and Elheparin in 20 per cent albumin. All have the same effect on the anti-D reaction in sodium chloride but in 20 per cent albumin with AB serum, heparin, liquemin, thrombophob, and heparin vitrum cause an increase in the reaction, thrombocid causes a decrease and Elheparin has no effect.

It was early discovered that heparin interferes with the action of complement[104]. However, cellulose sulphate, polyvinyl sulphate[105] and high molecular weight dextran sulphates[106] are much more effective anticomplement agents than heparin. The overall factor appears to be 'the charged surface area available for interaction of globulin components of complement'. The property is one shared by many high molecular size acids and bases, including germanin. Polysaccharide sulphuric acid esters prevent the fixation of C_1 and C_2 as well as the action of C_3 of complement[107]. The combining site of the anticoagulants for complement, according to Klein[108], is to an unknown

complement factor, CM. C_2 is bound to red cells only when CM and possibly other factors are present. The complementary function of guinea-pig serum poisoned by heparin or liquoid is restored not only by addition of complement inactivated by other means, but also by the addition of egg-white and gum arabic. Hence, the inhibition is a blocking action in which the structures are preserved, and it can be removed by other protein, including protein in plasma and serum. In specific blood group reactions of isoagglutinins and agglutinating or conglutinating Rh antibodies, the heparin and heparinoids may increase or decrease intensity of reaction, depending on blood, heparin and diluent[109]. Heparin and chondroitin sulphate can participate in various fixation tests such as the latex fixation test[110] for rheumatoid arthritis.

BIOLOGICAL REACTIONS OF HEPARIN AND HEPARINOIDS

The ability of heparin and heparinoids to complex with organic bases and proteins results in many significant biological effects. An interesting model for the biological effects of heparin as an ion exchange and adsorption agent is indicated by the data of Palos and Kocsan[111] and others. Leuco-methylene blue undergoes oxidation more quickly in an anaerobic system in the presence of heparin, as the dye is adsorbed onto the surface of the heparin and then becomes more sensitive to oxidative influences.

Viruses and Lower Forms of Life

In 1942, Cohen[112] found that heparin, hyaluronic acid and chondroitin sulphuric acid precipitated plant viruses, including tobacco mosaic virus, and in this way several viruses were obtained in crystalline form. In tissue culture, heparinoids show a potent reversible inhibitory effect on the early interaction of *Herpes simplex* virus and cells[113], with a minimum inhibitory concentration of 1 μg polyvinyl sulphate/ml. Effects have also been observed with encephalomyocarditis. Heparin has been identified as a component of the virulence-enhancing factor of hog gastric mucin.

Heparin at high concentrations[114] stimulates and later inhibits pseudopod formation in amoeba proteus. It is reported to have a comutagenic effect with x-rays[115] on *Streptomyces globisporus*, to have various effects on growth of plant seedlings, and to cause impairment of regeneration in planarians[116]. With frogs and invertebrate eggs, heparin reduces the number of dividing cells obtained by pricking[117] and it blocks mitosis[118]. Heparin, but not chondroitin sulphuric acid, stimulates the growth of tadpoles[119].

Tumours and Tissue Culture

Heparin causes a reduction in mitotic index and a decrease in tumour volume for Ehrlich ascites tumours in mice[120]. Polyethylene sulphonates having molecular weights between 15,000 and 35,000, also have antineoplastic activity in mice against ascites tumours, carcinomas and leukaemias[121]. Heparin has been found to reduce markedly the number of pulmonary metastases in rats receiving intravenous Walker 256 carcinoma cells[122]. Evans blue in the presence of a sulphated pectin entered tumour cells but not normal tissue cells[123]. The anti-immunochemical action of heparin has been used successfully for tumour heterografts[124,125].

Fischer's initial interest in heparin was aroused in 1928 when he found that it affected growth and differentiation of cells in tissue culture[292]. Mucopolysaccharides markedly increase aggregation of chick embryo cells which have been dissociated with trypsin, the decreasing order of activity being hyaluronic acid>chondroitin sulphate C>chondroitin sulphate A >heparin and carboxymethylcellulose. Heparin has no effect on strain L cells, apparently because it enters the cells and is then degraded[126]. Heparin also inhibits the cytochrome oxidase and succinic dehydrogenase of cultures from malignant tumours[127]. There appear to be significant differences in permeability for heparin of cells of different origins. Paff, Sugiura, Bocher and Roth[128] concluded that the ability of heparin to inhibit mitosis is due to interference with the metabolism of nucleoproteins, as in hanging drop tissue cultures, fibroblasts showed marked accumulation of ribonucleoprotein. When heparin is added to nuclei of rat liver, kidney or brain, there is a release of gel due to the release of DNA from nuclei[129]. Heparin also causes a dramatic fading of nuclei of ascites cells when they are freeze-thawed[130]. The injured plasma membrane allows the entrance of heparin resulting in dispersion and loss of the usually insoluble nucleohistones. DNA synthesis with parenchymal liver cell proliferation in normal adult rats[131] is increased by heparitin sulphate> heparin>chondroitin sulphate B>chondroitin sulphate A and chitin> polystyrene sulphate (no activity). Heparin and heparinoids prevent the inactivation, by a proteinase, of ribonuclease by liver homogenates. Heparin prevents the toxicity of human sera to mouse cells in tissue culture[132].

Effects on Blood Cells

A number of workers have reported that heparin causes thrombocytopenia[133]; the effect is transitory and is probably due to the temporary clumping of platelets which are trapped in the capillaries, with the result that thrombocytopenia is much more marked in capillary blood[134]. Cellulose, like dextran, glycogen, pectan, mannan and xylan, causes slight leucopenia, marked increase in sedimentation rate, marked rise in body temperature, and no change in blood or respiration. Cellulose sulphuric acid ester has no effect on the sedimentation rate and only slightly increases the body temperature while xylan sulphuric acid ester causes a pronounced leucocytosis, but no change in sedimentation rate or body temperature. Cellulose glycollic acid ether causes a leucocytosis but no change in the other parameters[135]. Dextran sulphate of molecular weight over 40,000 causes platelet and white cell agglutination whereas lower molecular weight dextrans have little or no effect on blood cells[136]. The difference is probably due to difference in nature and behaviour of non-ionic complexes formed with plasma proteins. The larger molecular weight group forms complexes with fibrinogen and α-globulin, and these are insoluble under physiological conditions and interfere with suspension stability of formed elements. In rats, heparin produces a leucocytosis. Cortisone and hydrocortisone cause a lymphopenia, which is prevented by heparin and dextran sulphate[137,138]. It is remarkable that heparin exerts this effect several hours after the injection of the steroids. In calves, heparin produces a lymphocytosis due to mobilization of lymphocytes[139].

Permeability and Wound Healing

The addition of heparin to the isolated rat diaphragm previously loaded with rubidium 86 results in change of the inflow of rubidium[140]. Heparin has a permeabilizing 'action' which appears to be due to mobilization of bound potassium and this can be related to a reduction of calcium in the perfusion solution. Karasek and Mourek[141] conclude that heparin depresses oxidative processes through an effect on cell permeability; it is possible that it adsorbs certain substances such as potassium chloride or acetylcholine. In extracorporeal dialysis[142], heparin causes an apparent decrease of 60 per cent in plasma calcium concentration. Heparin also forms a chelate-like compound with calcium ions[143]. This process is usually unimportant as heparin absorbs a maximum of only 5 per cent of its weight of calcium, but with intradermal, intramuscular or subcutaneous administration, heparin may deplete the capillary walls of calcium and cause them to become fragile. Heparin also decreases wound strength 6–14 days after operation in some experiments[144] and may delay the union of fractures in bone repair[145]. Heparin, but not chondroitin sulphate or hyaluronic acid, in tissue culture increases the amount of bone resorption in the presence of suboptimal concentrations of parathyroid extracts, and thus may be a cofactor in bone metabolism[146].

Endocrines and the Kidney

Pituitary extracts contain some basic protein which may interfere with heparin. Although ACTH and heparin have similar effects on endocrine weight, body weight, protein metabolism, lipid metabolism, sludged blood, anaphylaxis, dispersibility phenomena, and peripheral eosinophilia[147], heparin prevents the eosinopenia and lymphopenia due to ACTH and cortisone[148]. It has no effect on the depletion of adrenal ascorbic acid by ACTH, sodium salicylate, adrenaline or histamine, or by cold exposure[149,150]. In contrast, protamine blocks the depletion of adrenal ascorbic acid by ACTH.

Heparin has pronounced renal effects[151–153]. In healthy individuals, the diuresis test before and after intravenous injection of 50 mg of heparin shows a moderate, but not constant, increase in diuresis, and heparin inhibits the antidiuretic effect of pitressin. In trained unanaesthetized dogs, 50 mg of heparin intravenously causes a three or four fold increase in potassium excretion within 30 minutes, and this persists for 1 or 2 hours. This is not due to an effect on aldosterone and may be related to its potassium binding power. When heparin is given for longer periods of time, pronounced effects of heparin on aldosterone may, however, be seen[13,154]. Heparin, thrombocid and other heparinoids with no anticoagulant action, give increased urinary output of sodium chloride and water in patients with all types of oedema. There is no reduction in renal excretion of 10-hydroxycorticosteroids but there is almost complete suppression of aldosterone output in patients with secondary hyperaldosteronism. The increase of sodium output is much greater than is obtained with mersalyl or chlorothiazide in high dosage.

The hypotensive effect of heparin in hypertension[155,156] has characteristics which make it different from other hypotensive agents, and also different from other biological effects of heparin. Keller[157] reported that prolonged treatment with heparin (100 units/kg subcutaneously, three times weekly)

resulted in a lasting decrease of blood pressure to normal levels in rats and in patients with essential hypertension. The fall in blood pressure is a slow development and not an immediate effect in both animals and man, and this eliminates a direct dilator effect. Conditioning with large doses of heparin appears necessary and the fall in blood pressure is related to a mast cell discharge or effect.

Pharmacodynamic Effects

The ability of heparin to complex ions and simple bases is important in various pharmacological preparations. The decontraction or spasmolytic action of the isolated atropinized guinea-pig ileum after histamine contraction, however, is due to phenol and cresol in heparin solutions and not to heparin itself[158]. Green, Day and Roberts draw attention to the fact that heparin preparations contain more amino compounds than can be accounted for by the glucosamine[159], and it is these which cause the contraction of guinea-pig ileum. The contraction is different in shape from that of histamine, is not prevented by antihistamines, and is similar to that of polypeptides. Heparin also inhibits trypsin release of bradykinin from serum[160].

Heparin[161] and desulphated heparin[163] inhibit the contractions of frog-heart, nullify the systolic contracture caused by high calcium concentrations and counteract the effects of potassium chloride and acetylcholine[162]. Heparin also results in rapid recovery after cooling[164] and prolongs survival[162] of the isolated guinea-pig auricle.

Heparin[165] accelerates pigment formation from DOPA *in vitro* as well as when it is applied intracutaneously in rabbits. This probably explains the occurrence of pigment in urticaria pigmentosia. Unghvary, Farkas, Hovanyi and Farkas[166] report that heparin intravenously causes a reduction in the catecholamine level to about one-third of its original value, and inhibits the fluorescence of noradrenalin *in vitro*. Heparin, dextran sulphate, treburon, chlorazol fast pink, germanin[167] but not dextran, liquoid or negathol[168] antagonized curare in the rabbit sciatic-anterior tibialis preparation. Heparin decreases the toxicity of digitalis, ouabain, congo red and crude cobra venom[169,170] and reduces ouabain toxicity on the isolated rabbit auricle and cat papillary muscle[171]. Panasewicz[102] made the interesting observation that heparin reduces the excitability of vessel chemoreceptors to potassium chloride in isolated perfused limbs of the cat.

The first clinical trial of heparin in 1934 left an impression that heparin had an effect on peripheral blood flow. However, Zahn[173] did not find an effect on peripheral vessels in hypertensive and sclerotic patients. In Raynand's disease[174], heparin has been reported to cause increased skin temperature, pulse frequency, and a marked increase in arterial supply, together with a decrease in duration and severity of induced ischemic attacks[175]. Thrombocid is a vasodilator drug. Gilbert, Fenn and Nalefski[176] reported in 1949 that heparin and dicumarol have a direct coronary vasodilatory effect, but Patel and Patel[177] showed that treburon is a vasoconstrictor of the coronary vessels. In the Shorr and Zweifach test with the rat mesoappendix for vasomotor activity on arterioles and capillaries, heparin and heparinoids[178] show VDM activity (chondroitin sulphate and α-heparin$>$treburon and β-heparin$>$hyaluronic acid).

The heparinoids have actions as antihistamines. Selected cases of acute hay fever and asthma were helped by intravenous heparin, particularly for the acute attacks. Sufficient clinical improvement was observed to warrant further studies that heparin may provide protection against endogenous histamine. The combination of basic antibiotic and heparin is valuable[179], possibly due to reduction of toxicity of the antibiotic. While heparin has no effect on the action of histamine on the cat blood pressure, it suppresses histamine-induced gastric acid secretion in Heidenhain pouch dogs[176,180]. Chondroitin sulphate reduces the number of gastric ulcers in the Shay rat[181] but polyethylene sulphonates of molecular weights between 15,000 and 35,000, as well as other heparinoids, produce gastro-intestinal ulcers on chronic administration[121].

The vasoconstrictor action of shed blood tested by perfusion of the rabbit ear is markedly decreased by heparin and this was known long before the identification of the vasoconstrictor activity as serotonin (5-hydroxytryptamine). Heparin inhibits the release of both 5-hydroxytryptamine and a polypeptide[182] from platelets into plasma, and *in vitro* there is mutual antagonism between heparin and 5-hydroxytryptamine[183]. Heparin effectively antagonizes the effect of serotonin on pulmonary vascular bed and bronchial wall musculature and prevents the symptoms of 5-HT release[184] in pulmonary embolism, in the cardiopulmonary by-pass, in experimental burn injuries[185] and in carcinoid tumour.

Protective Effect Against Trauma and Toxins

Pulmonary resuscitation experiments in dogs indicate that heparin is valuable because it greatly prolongs the period during which blood can be moved[186]. Both heparin and depolymerized sodium hyaluronate protect rats and rabbits from the effects of compression[187,188]. On the other hand, heparin has no effect on ischemic compression shock[189]. Heparin increases the survival time of animals receiving lethal burns and tourniquet shock[190,191] as well as the speed of repair mechanisms after burns. With trauma (by tumbling in the Noble–Collip drum), heparin diminished and retarded mortality[192]. Heparin delayed the convulsions and death in mice receiving cardiazole but did not affect the mortality incidence[192] nor have any effect on shock from insulin hypoglycemia in rats. Karasek and Mourek[141] report that heparin gives an increased resistance to nitrogen hypoxia and to cardiazol convulsions, but heparin has no influence on respiratory and circulatory reflexes resulting from breathing pure carbon dioxide. In anaesthetized dogs, heparin administration[193] appears to prevent the production or the release of lactate during hypoxia. Higginbotham[14] has shown that the ability of heparin to combine with toxic bases results in one of its important biological properties—protection against lethal agents. In mice receiving $1{\cdot}6 \times LD_{50}$ of Russell Viper Venom, the 50 per cent protective dose was: heparin 0·55, heparin monosulphate 4·0, chondroitin sulphate B 6·7 μg/g with hyaluronic acid, chondroitin sulphate A and C, inactive. The protection given by mucopolysaccharide was much greater than that with cortisol or immunization. As a result of the reduction of toxicity of neomycin in mice with heparin, it was possible to give a higher dose of neomycin with high survival with a lethal staphylococcus injection[14].

Inhibition of Anaphylaxis, Sensitivity Reactions, Inflammation

In 1926, Keyes and Strauser[194] demonstrated that heparin inhibits anaphylaxis in pigeons, and in 1928 Van de Carr and Williams[195] showed that heparin not only inhibits anaphylactic shock in the whole guinea-pig but also inhibits the response of the isolated Dale preparation and the bronchospasm reaction of Koessler and Lewis. While heparin may interfere with the reaction of antigen and antibody[47], it may act by interfering with some of the other steps in development of the anaphylactic symptoms. For example, Marx, Bayerle and Skibbe[104], emphasizing that anticoagulant activity is not responsible for the inhibition of anaphylaxis, drew attention to the possible relation with anticomplementary activity. Johansson[196] reported later that pretreatment of rabbits with heparin prevented thrombocytopenia and the release of 5-hydroxytryptamine from sensitized blood cells *in vitro* and *in vivo* and suggested that it acts by preventing absorption and release of 5-HT by platelets[197]. Rothschild and Rocha e Silva[198] showed that heparin inhibits formation of anaphylatoxin in rat serum, diminishes anaphylatoxin shock and abolishes reversed anaphylactic shock.

The inhibition by heparin of anaphylactic and sensitivity phenomena has been observed as an adjuvant effect of other uses of the drug in patients with cutaneous allergic reactions[199], severe asthma[200], bronchopulmonary disease[201], infected otitis[179], and in sensitivity conditions studied in animals, for example rat glomerulonephritis produced by successive injections of horse serum, rabbit nephritis due to nephrotoxic serum[202], rabbit immune nephritis by injection of heterologous antikidney serum[203], rabbit glomerulonephritis by injection of γ-globulin[204], rabbit uveitis from antigen[205], and the Arthus phenomenon[206]. With the latter, heparin attenuates the reaction so that the symptoms disappear without leaving traces. The effect of heparin on allergic phenomena may explain why there are relatively few instances of sensitization during heparin and heparinoid therapy[207,208].

The varied activities of heparin in relation to ground substance, pinocytosis, and biological ion exchange suggests important anti-inflammatory functions[209]. When inflammation is produced from multiple subcutaneous injections of egg-white in rabbits with biopsy of connective tissue, it is observed that heparin, chondroitin sulphate A, heparitin sulphate, sulphonated alginic acid, sulphonated chitin and chitosan, sulphopolyglucan (but not dextran sulphates) inhibit mononuclear cell exudation[210]. This effect is not reversed by protamine and not correlated with anticoagulant activity. Heparin[211] and dextran sulphate[212] inhibit activation of the globulin which causes increased capillary permeability, as part of the inflammatory exudate process, but heparin[213] has no effect on the sticking of white cells to endothelium in inflammation. It is interesting that Branceni *et al.*[214] have developed a heparin analogue, β-naphthoyl-heparamine, which they find may be given intra-articularly to give a reproducible inflammation. This may be used to assay the anti-inflammatory action of salicylates, phenylbutazone, and hydrocortisone.

Antilipaemic Activity

There is a marked decrease or clearing of lipaemia[3,11,22,258] when these compounds are injected into lipaemic subjects. This is readily demonstrated

when two plasma samples are examined from a subject after a fat meal, the second sample taken five minutes after injecting heparin. The creamy, cloudy appearance of the first sample due to the chylomicra has disappeared in the second. This is accompanied by a marked increase in unesterified fatty acids and a decrease in total serum glycerides. The most important change is the marked loss of all lipids except unesterified fatty acids from plasma proteins (particularly the S_f 20–400 fraction). Electrophoretically, there is a decrease in the area of the β-globulin peak, an increase in the α-globulin peak, and the formation of a new peak corresponding to the α_1-globulin or albumin peaks, or with even greater mobility. The effect is due to the appearance in plasma of an enzyme, lipoprotein lipase, which hydrolyses the triglyceride moiety of lipoproteins. The resulting unesterified fatty acids are adsorbed onto albumin and pre-existing lipoproteins. The injection of heparin into the fasting individual likewise results in the appearance in plasma of lipoprotein lipase, as may be shown by testing the plasma by adding it to lipaemic plasma, triglycerides with α-lipoproteins, or egg-yolk lipoproteins. Albumin is required to adsorb the fatty acid products, which otherwise quickly stop the reaction. Perfusion experiments have shown that heparin releases the enzyme from isolated hind limb, skin, subcutaneous tissue, lung, kidney and intestine but not from the liver. Heparin appears to be associated with the enzyme, at least to the degree that removal of heparin decreases the stability of the enzyme. The enzyme is a normal constituent of many tissues but is released into blood only by special agents. Lipoprotein lipase can be produced by the release of endogenous heparin in peptone and anaphylactic shock and by all heparinoids and the high molecular weight inorganic ions such as phosphotungstate, phosphomolybdate and polymetaphosphate.

ANTICOAGULANT ACTIVITY

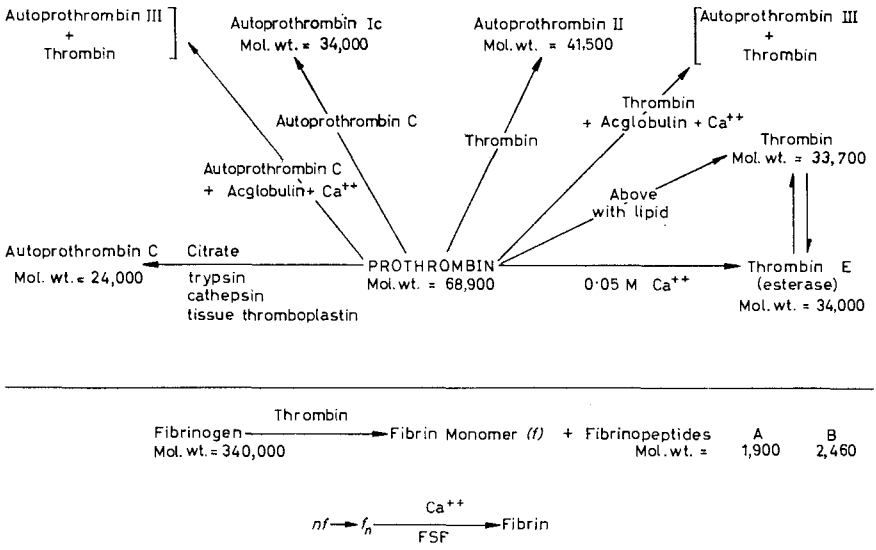
Blood Coagulation

The main chemical processes taking place when blood clots have recently been completely identified by modern biochemical methods. These reactions (shown in *Table 3.3*) involve the cleavage of prothrombin and of fibrinogen to smaller molecular weight proteins, and this is followed by polymerization of the fibrin monomer to form the protein of the gel or clot. The cleavage of prothrombin yields a number of proteins of lower molecular weight and the yield of these varies with the conditions. In addition to several substances which have thrombin activity, other protein products have the ability to split prothrombin, i.e. they have autocatalytic activity and are called autoprothrombins. Thrombin hydrolyses several basic polypeptides from the side of the long cigar-shaped molecule of fibrinogen. With the net loss in charge, the molecules then associate to form a polymer. This is further cemented together by calcium ions and FSF (fibrin stabilizing factor) through disulphide linkages.

These basic chemical reactions occur when blood clots (the Fuld–Morawitz hypothesis). However, the blood clot is a complex structure derived from the blood, in which cells and fluid matrix are held together and to the wall of the container by fibres of the protein, fibrin, together with disintegrated platelets. The rate at which sufficient thrombin is formed to provide an

effective fibrin clot is modified by various 'factors'. The rate of clotting of blood is measured as the coagulation time, or length of time for a clot of fibrin to appear. One might consider the base line as the longest time taken for clotting, without the addition of inhibitors. This was achieved by Jaques, Fidlar, Feldsted and Macdonald[217] as follows: when blood is taken carefully in a syringe coated with silicone and the central portion transferred

Table 3.3. Protein chemical reactions in blood coagulation[215] (as shown by chemical criteria)



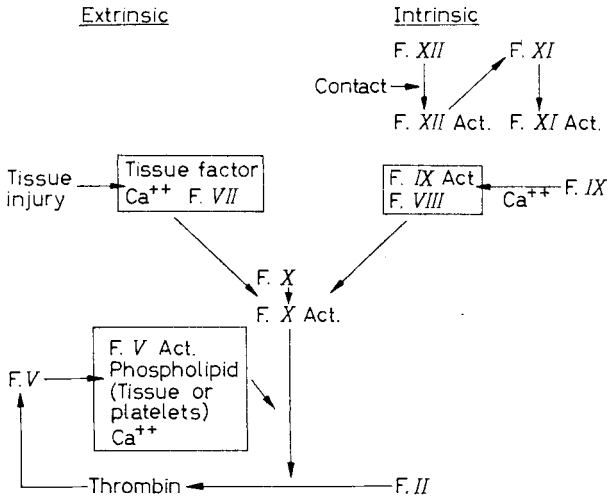
to tubes coated with silicone, it takes several hours to clot. When the blood comes into contact with glass, it clots in 5 minutes. If a tissue extract is added to the blood, it clots in 12 seconds—an accelerated coagulation time.

For convenience of duplication, it is common to add the blood to a decalcifying agent and obtain plasma for the test, which can be recalcified as required. Plasma plus calcium plus tissue extract (thromboplastin) constitutes the prothrombin time test procedure introduced by Quick. The prothrombin time test involves several steps—preparation of plasma, mixing of reagents, and timing of conversion of fibrinogen to fibrin. More information can be obtained when these are separated further by timing the conversion of a standard fibrinogen substrate at various times after mixing plasma and activation mixture. This constitutes the two-stage principle. All these tests are dependent on the appearance of sufficient thrombin in a sufficiently short time to provide a fibrin clot. The rate of clotting is affected in the different tests by material from tissues (the extrinsic thromboplastin which is cephalin-protein), by blood cells (platelets, leucocytes, red cells), and by materials in plasma (the intrinsic thromboplastin, the formation of which is aided by surface contact activating the Hageman factor and antihaemophilic globulin).

By use of suitable factor preparations to modify the coagulation time, a great variety of tests has been developed. These have been particularly

useful for the differential diagnosis of hereditary haemorrhagic conditions (e.g. haemophilia). Because of the number of partly characterized activities and corresponding tests and the great number of workers actively engaged in their study, there has been confusion due to multiplicity of terms. In 1954, an International Committee was established by the workers themselves, and has provided a common denominator for the many synonyms by the use of Roman numerals for those influences occurring in plasma. This provides a means of communication and even a shorthand description, until such time as complete chemical identification makes possible a logical system of nomenclature[218]. Blood coagulation schema commonly show the relationships of these influences in diagrammatic form (*Table 3.4*). These influences (com-

Table 3.4. Factors involved in the production of thrombin in plasma coagulation tests (used in the differential diagnosis of haemorrhagic disease or conditions)



F. *II* = Factor *II* = Prothrombin activity (=source of thrombin)
F. *V* = Factor *V* = Ac-globulin activity
F. *VII* = Factor *VII* = Proconvertin activity
F. *VIII* = Factor *VIII* = Activity corrective in haemophilia A
F. *IX* = Factor *IX* = Activity corrective in haemophilia B
F. *X* = Factor *X* = Activity corrective in haemophilia C
F. *XI* = Factor *XI* = Activity corrective in SPCA deficiency
F. *XII* = Factor *XII* = Activity corrective in Hageman trait

monly called factors) represent a great diversity of partly characterized materials which may be surface forces, lipids, enzymes, or products or modifications of the prothrombin molecule. Some of these factors are now identifiable as plasma proteins. Others probably represent an activity shared by various biochemical substances. Thus, the thromboplastin activity in tissue extract has long been known to be associated with a cephalin-protein compound[219]. Trypsin[220] and hyaluronic acid[221] also possess this activity. Since the latter is in closer proximity to the blood when a vessel is damaged, it may be of greater physiological significance. Presentation of the sequential effect of these factors in producing thrombin activity in coagulation tests, as in *Table 3.4*, is extremely useful in correlating information on the tests. Such diagrammatic schema should not be taken for representations of chemical reactions comparable to those depicted in *Table 3.3*.

Types of Anticoagulants

Anticoagulants are by definition agents which slow or prevent the coagulation of blood. They are divided into two types, direct and indirect anticoagulants. This division is based on whether they produce inhibition of blood coagulation when added directly to blood *in vitro* (the direct anticoagulants), or whether they must be first administered to the animal or patient to produce inhibition of coagulation of the blood *in vitro* (the indirect anticoagulants). These may be further subdivided.

Direct anticoagulants

(1) Those which combine with or inhibit a single component of the coagulation process. For example, EDTA (ethylenediaminetetra-acetic acid, sodium salt) complexes and effectively removes calcium ion. *Hirudin* forms a stable inactive complex with thrombin through its carboxyl groups combining with the amino groups of thrombin and blocking the active sites of the enzyme. *Protamine* and *basic dyes* appear to be competitive inhibitors for thromboplastin (intrinsic and extrinsic). *Fibrin* (Antithrombin *I*) adsorbs large amounts of thrombin.

(2) Those which combine with or inhibit several components of the coagulation process, e.g. sodium, potassium and ammonium *oxalate* and *citrate*. Oxalate not only precipitates calcium but the resulting insoluble salt absorbs components of the prothrombin complex. Citrate complexes and removes ionic calcium and also combines with plasma proteins (components of the prothrombin complex and fibrinogen) and modifies their properties. *Heparin in concentrations greater than* those which produce an *anticoagulant* effect in blood and plasma, combines and modifies many of the proteins of the coagulation system—for example, fibrinogen.

(3) Those which do not inhibit coagulation directly but on being added to plasma produce an inhibitor. *Heparin in concentrations sufficient* to exert an anticoagulant effect in blood and plasma is the best example. This is a very poor inhibitor when added to purified coagulation systems, but with a special plasma protein, the so-called 'heparin cofactor', it gives rise to an active anticoagulant.

These definitions of direct anticoagulants are based on the effect *in vitro*. From the standpoint of pharmacology and therapeutics, a direct anticoagulant should act *in vivo* to give a maximum inhibition of coagulation as soon as it is completely mixed in the circulation with the blood pool. Up to the present, such an anticoagulant has not been described. A direct anticoagulant that takes an hour to produce its maximum effect *in vivo*, must be acting as an indirect anticoagulant.

Indirect anticoagulants

(1) Heparin-liberators. *Peptone* and *specific antigen* in the sensitized animal are the most effective liberators of heparin from mast cells. Many histamine liberators also liberate heparin but since many are strongly basic amines, they may combine with and mask the liberated heparin. *Commercial heparin* may have some action as a heparin-liberator[216].

(2) Liberators of heparinoids and other mucopolysaccharides. These

substances may be liberated from the arterial wall or gastric mucosa. They show a slight effect on coagulation time of blood but give an increased titration value for many heparin tests, for instance nitrilotriacetic acid (NTA)[222].

(3) Liberators of antithrombin and/or heparin cofactor. The latter proteins appear to be secreted by the liver, and result in hypocoagulability in thrombin titration tests and increase the sensitivity of the coagulation time and other tests to heparin. Papain releases antithrombin[223].

(4) Coumarin and indanedione derivatives. These are fixed in the liver and either directly or through a metabolite interfere with the production of prothrombin protein and its active derivatives[5]. Examples are dicumarol, warfarin and phenylindanedione.

Coagulation Time Tests

The majority of studies have been based on the measurement of the clotting time or coagulation time. However, there are many standard methods of measuring this and since they measure separate and significant parameters, it is essential to identify the type of determination used.

The most common tests are: (1) *Clotting time of whole blood*; (2) *clotting time* of whole blood collected and measured *in silicone*; (3) *prothrombin time* (*Quick*)—clotting time of recalcified plasma with added thromboplastin (tissue extract); (4) *plasma time* (*Howell*)—clotting time of recalcified plasma; (5) *plasma time* (*Fischer*) with chicken plasma—this is not decalcified and therefore calcium is not added; (6) *thrombin time*—clotting time of decalcified plasma with added thrombin; (7) *two stage tests*—modifications of the above tests in which the final measurement is made as the coagulation time of a separate fibrinogen solution on the addition of an aliquot from the test. Thus there are the two-stage prothrombin time, and two-stage plasma time tests; (8) *thrombin generation test*—fibrinogen solution is standardized to give values as units of thrombin; (9) *thromboplastin generation test*—appearance of thromboplastin activity as measured by an aliquot of the sample shortening the plasma time or similar test; (10) *whole blood clotting time* (*in vivo*)—resulting from the administration of the test substance to the subject; (11) *TAME*—hydrolysis of tosyl-arginine-methyl-ester by thrombin; (12) *thromboelastogram*—recorded with Hartert thromboelastograph.

The coagulation time is a rate function, with its value decreasing as the concentration of coagulant substances increases, and increasing as the concentration of inhibitors increases. On an empirical basis, it has been found that there is a logarithmic relation usually between the concentration of any coagulant or any anticoagulant and the coagulation time[224], and perhaps more important, that the variation in the values with the same amount of reagent (the deviation) bears a constant relationship to the absolute value. This is particularly useful in graphic reporting of experiments involving coagulation times, since on ordinary plotting, the variability at the long values makes graphic representation bizarre, yet fails to show the significant changes occurring with the short coagulation times. Expressions have been derived for the relationship between log clotting time and concentration of components for thrombin and prothrombin[224], and for heparin[225]. As shown by Jaques and Mustard[226], in considering the influence of heparin or any other factor on blood coagulation, it is important to remember that the

apparent activity of any factor depends upon the base line. They showed that when the coagulation time of plasma is prolonged by increasing the concentration of sodium chloride, then the apparent effectiveness of heparin will be markedly increased. This is related to the fact that a logarithmic relationship is involved and the two variables together will have a much greater effect than the sum of the individual effects.

As shown in *Figure 3.2*, the clotting of heparinized blood is very sensitive

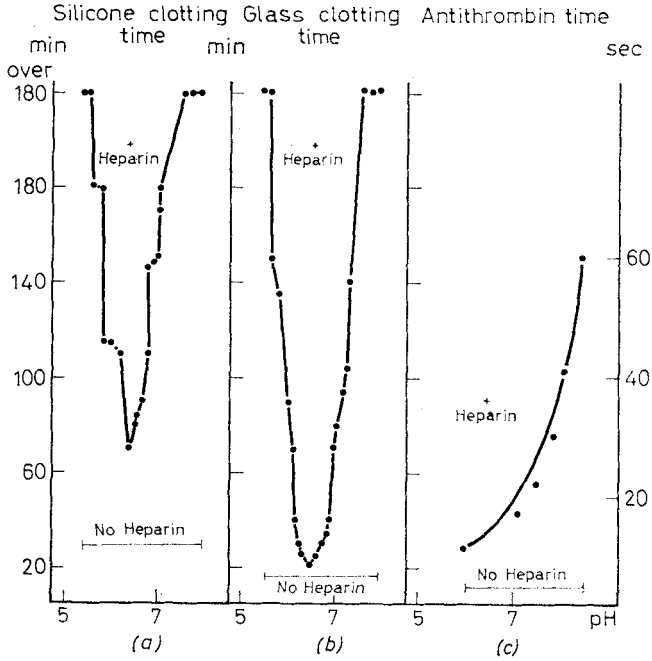


Figure 3.2. Effect of pH on anticoagulant action of heparin[227, 228]:
(*a*) clotting time of whole blood in silicone;
(*b*) clotting time in glass;
(*c*) antithrombin time

to change in pH. At pH 6·0–6·6, heparinized blood (incoagulable at pH 7·8) clots almost as rapidly as without heparin. With regard to temperature, clotting times are shortest at 20°C, although the effect of temperature is not the same for different tests.

Anticoagulant Action of Heparin and Heparinoids

The basic facts about the anticoagulant action of heparin are that (*i*) 1 to 10 μg is effective in delaying the clotting of 1 ml. of blood or plasma; (*ii*) this activity is dependent on the presence of a plasma factor, the cofactor[229–231]. The most exact statement on the nature of the anticoagulant action of heparin has been made by Brambel, Corwin and Capone[232]: 'A characteristic property is imparted to the environment when heparin and its cofactor are present. The differentiation between chemical combination or alteration between constituents cannot be made with any degree of certainty

at this time. Until evidence is produced to the contrary, a combination is presumed and the term "heparin-cofactor complex" is coined to denote biochemical characteristics of plasma after interaction of heparin with its cofactor.'

In defining anticoagulants above, it was emphasized that heparin in concentrations of 0·1 mg/ml. and over, shows general inhibitory effects and this has led to much confusion in the literature. The important problem is the nature of the action of the heparin-cofactor complex which provides the unique high activity of heparin. In analysing the inhibitory effect of heparin in plasma systems, the essential constituent is the cofactor and its concentration is an unknown limiting variable in many investigations[233].

While the heparin anticoagulant shows antithrombin activity, it requires only 0·003–0·01/mg of heparin/ml. of plasma to inhibit the formation of thrombin, yet it requires 0·1–0·4 mg to inhibit the action of the formed thrombin[220]. Hence the anticoagulant effect is due to inhibition of thrombin formation. Further analysis indicates that the anticoagulant inhibits (in order of increasing effectiveness): thrombin, thromboplastin, Factor *V*[232], thromboplastin generation[235], Factor *IX*[236], and the contact effect (Factor *XI*)[237]. The interaction of heparin and platelets is complicated. Heparin inhibits aggregation of platelets by thrombin but not by ADP[238] so that it only partly inhibits platelet contact agglutination and formation of intrinsic thromboplastin. Platelets contain both an antiheparin component (Platelet Factor 3) and a thromboplastic factor[239]. Heparin is a more effective inhibitor of plasma clotting in glass with a platelet-rich plasma. On the other hand, the highest degree of inhibition (most sensitive test for heparin—0·25 μg/ml.) is achieved with platelet-free plasma in silicone.

While the antithrombin produced when heparin is added to plasma is quantitively the smallest part of the anticoagulant activity of heparin, it is closest to the indicator system or the conversion of fibrinogen to fibrin by thrombin and has received the most study. It is a reasonable assumption that this may be taken as a type activity for the anticoagulant action of heparin, although it must be emphasized that the use of this analogy has dangers. 'Antithrombin' is used to refer to what happens to thrombin when it is incubated with serum or defibrinated plasma and later added to fibrinogen for estimations. It is also used for changes in the clotting time of a mixture of fibrinogen and thrombin with added plasma. Disappearance of added thrombin occurs without heparin, but the addition of heparin results in more rapid disappearance. Both phenomena depend on heat-labile plasma proteins and while Astrup and Darling[240] were able to distinguish the two activities on the basis of destruction at 65° and 56°C respectively, separation of the two by fractionation procedures has been difficult.

Heparinoids may possess one or several of the anticoagulant effects listed previously. The general impression gained from the literature is that different heparinoids possess many different anticoagulant properties to differing degrees, and the failure to appreciate the varied possibilities has resulted in a limited investigation of the anticoagulant properties of a given heparinoid. The one attempt to investigate the action of a heparinoid in detail is that of Pulver[241,242] whose ideas are summarized thus: (1) intravenous injections of heparinoid cause anticoagulant effects similar to those of heparin;

(2) heparinoid does not combine with the heparin cofactor; (3) the antithrombin activity in a plasma sample can be raised to a constant final value by the addition of increasing amounts of heparinoid; and (4) therefore heparinoid acts by mobilizing plasma protein-bound heparin, which he terms heparinogen. These fairly simple concepts provide the only explanation of the anticoagulant action of heparinoids which agrees with considerable information available. However, Pulver's experiments do not provide direct and complete evidence of this, partly because the most important variables to control are in the unknown mixture known as plasma.

Uses of Heparin as an Anticoagulant

Heparin was introduced to clinical use as an antithrombosis drug. On the introduction of oral indirect anticoagulants, these replaced heparin in the treatment of many patients with thrombosis, but the effectiveness of heparin as a direct anticoagulant has resulted in many other clinical uses. A survey of current clinical uses in the University Hospital, Saskatoon, showed in six months the following quantities of heparin used in units $\times 10^5$ (or approximate weight in grams): thrombophlebitis 10·3; arterial thrombosis 5·2; myocardial infarction 3·4; burns, gangrene, peritoneal dialysis 12·0; cardiac and related surgery 8·0; cardiopulmonary diagnosis (cardiac catheterization and blood gas analysis) 51·1; other laboratories 0·6; unassigned 9·7; total 99·7. This means the clinical use for treatment of thrombosis (in 25 patients) during this period was only 20 per cent of the total used, and 80 per cent was used in patients as an anticoagulant adjunct to diagnosis, irrigation or surgical repair.

Purified heparin is used as a substitute for oxalate and citrate. The amounts of common anticoagulants required to prevent coagulation effectively *in vitro* in mg/ml. are: potassium oxalate, 0·5; sodium and ammonium oxalates, 0·5; lithium oxalate, 0·3; Heller and Paul mixture, 0·5; sodium citrate, 2·0; sodium fluoride, 2·0; liquoid Roche, 0·3; novirudin, 0·5; chlorazol dye BKS, 0·5; heparin Connaught, 0·1; Heparin B.D.H., 0·1[243]. There are now available heparinized vacuum tubes which can be used for determination of plasma pH, plasma carbon dioxide, blood oxygen saturation[244]. With the introduction of rapid semi-automatic and automatic systems of microchemical analysis in the clinical laboratory, heparin appears to be the anticoagulant of choice. However, for blood glucose, preservatives are required for most of the anticoagulants if a longer period of time than $2\frac{1}{2}$ hours is to elapse after collection of sample[245]. The complexing properties of heparin should be taken into account when it is used as an anticoagulant, although generally the amount used is too small to affect clinical chemistry determinations. Heparin appears to be a source of error in the determination of plasma albumin on a basis of selective dye-binding[246].

Heparin is well known for its usefulness in the pharmacology laboratory in many animal experiments for preventing clotting during recording of blood pressure. The many manifold effects of heparin on hormones, cell permeability, activation of various enzymes, and uptake of ^{32}P by brain tissue, must be controlled in animal experiments when heparin is used as an anticoagulant.

The use of heparin in experimental exchange transfusion was pioneered

in 1938 by Thalhimer, Solandt and Best[247]. Its particular value in blood transfusion lies in the fact that it does not upset any ion balance of the blood and it is readily metabolized. It is even possible to give the anticoagulant to the donor so the anticoagulant is in the blood before it is drawn. It is therefore the most satisfactory anticoagulant for the exchange of large amounts of blood. Heparin has better tolerance and much fewer complications than any other anticoagulant.

Heparin has now had 20 years of use for total exchange transfusion in infants[248]. It is used universally with the various types of extracorporeal apparatus, such as artificial lung and artificial kidney. In these situations the blood is maintained incoagulable by the addition of heparin while the transfusion is continued, and at the conclusion of the procedure, the heparin in the circulation is neutralized either with protamine or polybrene. With the dialysis type of exchange[249], the regenerated cellulose is for all practical purposes impermeable to heparin, and neither dialysis nor ultrafiltration significantly influence the elimination rate of heparin. Clotting is not a common cause of difficulty with prolonged exchanged transfusions, but mechanical trauma in such systems results not in deposits of fibrin[250] but of fragmented red cells. The addition of glucose and other sugars to heparinized blood or plasma before use can result in clotting and corresponding reduction of platelets and coagulation factors in the circulation[251].

Heparin is widely used as an added reagent in coagulation tests with the hope that some parameter of coagulability will be indicated in patients, particularly those circulating thromboplastin. As the clotting time is increased, there is at least the possibility of increasing the sensitivity of the clotting time test to various coagulant factors. This constitutes the so-called *heparin tolerance test*, which was first popularized by de Takats[252] both *in vitro* and *in vivo*. The heparin tolerance test has been used with almost as many different types of coagulation end-points as have been involved in coagulation tests generally, usually with a simple series of different heparin or heparinoid concentrations. Systems in use are: blood, recalcified oxalated plasma, thrombin time, prothrombin time, and thromboplastin generation[253]. While the heparin tolerance test has been advanced particularly as a method of measuring the level of circulating thromboplastin, there is no evidence for this[254], nor that it reflects decreased concentration of heparinoids or heparin in the blood but rather the reverse.

HAEMORRHAGE AND THROMBOSIS

Spontaneous Haemorrhage

Delayed blood coagulation is not necessarily accompanied by haemorrhage. Many thousands of patients have received anticoagulants to render their blood incoagulable, without developing haemorrhage. In the clinical condition known as afibrinogenemia, the patient's blood is completely lacking in fibrinogen, and no bleeding occurs for most of the time. This means that the process of haemostasis, the prevention of haemorrhage as a normal physiological process, is not synonymous with blood coagulation. The key to the understanding of the development of haemorrhage with anticoagulants was the finding that while normal animals rarely develop spontaneous

haemorrhage when treated with moderate doses of anticoagulants, a large percentage of such animals die from spontaneous haemorrhage if exposed to stress[5]. This has made possible the rational study of spontaneous haemorrhage. Serious spontaneous haemorrhage can be produced experimentally in animals by various treatments shown in *Table 3.5.* The many different

Table 3.5. Production of spontaneous haemorrhage

Types of treatment

(1) Agents affecting vascular factor.
Stress:
formalin s.c., frost-bite, hypertonic saline i.p., sham operations, restraint, physiological saline daily s.c., electroshock, insulin convulsions, L.S.D., lysergic acid, picrotoxin, metrazole.

Procedures acting on or by the adrenopituitary axis:
ACTH, STH,
Salicylate, adrenalin, histamine,
Adrenalectomy,
Deoxycorticosterone.

(2) Platelet depleting agents—^{32}P, reserpine.

(3) Anticoagulants—dicumarol, phenylindanedione, heparin.

Occurrence of spontaneous haemorrhage

Types of treatment given	(i)	(ii)	(iii)
(i)	−	+	+
(ii)	+	−	+
(iii)	+	+	−

+ = Mortality of 30–100 per cent from spontaneous haemorrhage
− = Mortality < 20 per cent (usually < 1 per cent) from spontaneous haemorrhage

procedures effective have been listed in three groups. The first group consists of many stress procedures, injections of hormones and drugs, and adrenalectomy. The second consists of procedures which affect platelets—^{32}P and reserpine.

The third group consists of the procedures which affect the blood coagulation process or the administration of anticoagulants. A fairly objective criterion is a mortality from spontaneous haemorrhage of 30–100 per cent. Any of the treatments in the groups 1, 2 or 3 alone does not cause haemorrhage by this criterion. Even when several treatments in the same group (such as heparin plus dicumarol) are given, haemorrhage of this order of severity does not result. However, if two procedures which appear in different groups are given simultaneously, the result is fatal, i.e. 1 + 2, 2 + 3, or 3 + 1. The treatments used correspond to interference with (*i*) the living blood vessel wall, (*ii*) platelets, and (*iii*) the coagulation process which provides the enzyme, thrombin. Stress, adrenalectomy and hormones affect the composition and effectiveness of the vascular wall in holding against haemorrhage. A deficiency in platelet effectiveness is produced when platelets are decreased in number or composition. Anticoagulants interfere with the coagulation system, the formation of thrombin. These are three facets to haemostasis. The results demonstrate that *simultaneous interference with more than one facet of haemostasis is required to produce severe spontaneous haemorrhage* and that the spontaneous haemorrhage has *multiple causation.*

Haemostasis

The tendency to haemorrhage or thrombosis can be summed up in the formula:

$$\frac{\text{Vessel wall } (V) \times \text{Blood composition } (C)}{\text{Blood flow } (F)} = \text{Haemostatic efficiency } (K)$$

or

$$\frac{V.C}{F} = K$$

Changes in the complex phenomena represented by each of these terms will result in changes in the haemostatic efficiency. The significant values for K are K_t and K_h, the critical thrombotic value and the critical haemorrhagic value. When the product of $V.C/F$ reaches the value K_t at a certain point in the circulation, thrombosis will be initiated. When it falls to K_h at a certain point in the circulation, haemorrhage will result. Anticoagulants chiefly (but not solely) affect C (the composition of the blood) to decrease haemostatic efficiency. The three factors, vessel wall, blood composition and blood flow are not completely independent. The vessel wall component is affected by blood composition and blood flow, and *vice versa*, and situations arise where thrombosis and haemorrhage can occur simultaneously in the circulation of the same individual.

The physiological and pathological factors modifying these factors, and hence the haemostatic efficiency of the cardiovascular system, have been the subject of many experimental investigations and clinical observations. These are summarized in *Table 3.6.*

Table 3.6. Physiology of haemostasis

PASSIVE	ACTIVE
State of the Cardiovascular System	*Response to Vessel Injury*
Integrity of the blood vessel wall is determined by:	*Retraction and constriction of vessels*
	Mechanical: Cutting vessel removes stretch with shortening (isometric to isotonic contraction)
Nervous system: Cortex & mid-brain—limbic system & reticular formation—vasomotor centres→reflex vessel tone; pituitary→ACTH, etc.	Reflex: Segmental contraction of vessel
	Contraction by autopharmacodynamic agents: SVPx, adrenalin, serotonin, histamine released locally from platelets, adrenochrome from sympathetic endings
Nutrition: proteins, lipids, vitamins C & K, Na, K	Biochemical: Actin + Myosin (ATP)→Actomyosin
	Repair
Endocrines: STH, corticosteroids, oestrogens, etc.	Trapping of platelets: Exposed collagen traps platelets, and converts ATP to ADP, which aggregates platelets to loose, detached clumps
Haemodynamics: Blood pressure, blood flow, tissue fluid pressure	Viscous metamorphosis (V.M.): Thrombin formed in platelet atmosphere causes V.M. which gives firm attached clumps (haemostatic plug)
Blood composition: O_2, CO_2, pH, oncotic pressure, etc.	Clotting of blood: occurring in closed, sealed vessel gives final reinforcement

The haemostatic process is a complex interaction involving the blood vessel wall, platelets and blood coagulation. In the important vascular component can be distinguished passive and active elements. The relative

importance of the various components of haemostasis varies with the size of blood vessel and type of damage to the vessels. Clotting of blood is a significant but not essential or highly important feature of haemostasis. As appreciated by Bizzozero[255], the later haemostatic process is identical with the process of thrombus formation.

Effect of Heparinoids on Haemostasis

It can be demonstrated that anticoagulants delay the microscopic processes of haemostasis[256–258]. It was emphasized above that incoagulability of the blood does not necessarily result in spontaneous haemorrhage and that pure coagulation defects have little effect on haemostasis[259–261]. On the other hand, an increase in nonanticoagulant heparinoids in the blood has haemorrhagic proclivities[369]. The changes in the blood coagulation factors in leukaemia may be related to the mucopolysaccharide in the leucocytes. With an increased protamine titration in bleeding patients who show no defects by coagulation tests, it has been found possible to stop bleeding with intravenous toluidine blue and the protamine titration returns to normal regardless of platelet levels.

The relationship of heparin to haemostasis and haemorrhage is complex. Heparin does not cause haemorrhage because of its anticoagulant activity. Doses which produce incoagulability of the blood do not result in a haemorrhagic tendency and this was an essential observation for the introduction of anticoagulant therapy[2]. Excessive doses cause haemorrhage through interfering with myosin in vascular smooth muscle, since ATP and heparin are mutually antagonistic in haemostasis, for example, in the rat-tail[262].

Another haemorrhagic action of heparin appears to be on the basement membrane, since large doses in the whole animal cause leakage of red cells and platelets into the lymph[263]. These results can be reconciled by the distinction referred to in other sections of this review between free and bound heparin in plasma. Plasma-bound-heparin, while exerting an anticoagulant action, is not free to exert effects in tissues. Free heparin is able to do this and causes haemorrhage. Finally, disruption of haemostasis by anticoagulants occurs when there is additional interference with the state of the cardiovascular system, as with stress, causing the blood vessels to be leaky. Heparin can increase or decrease this latter effect by its interference with corticosteroids.

It is usually stated that heparin should never be used in bleeding, but one important exception to this is the bleeding due to primary hypercoagulability. Intravenous injection of heparin has produced dramatic cessation of bleeding[264]. Other conditions related to intravascular clotting which have been successfully treated with heparin are toxemia of pregnancy[265], the defibrination syndrome[266], and the verbrauchskoagulopathien or Sanarelli–Shwartzman phenomenon[267].

Thrombosis

This is commonly thought of as intravascular clotting. There is however an important difference between what happens to blood in a test-tube and what can happen to blood in the circulation, since in the latter, the blood is in constant movement or flow. In the test-tube, only what is already present

can appear in the clot and will be present in the proportions present at the beginning. In the circulation, other material can be brought and collected. The appearance and composition of a thrombus therefore depends on the vessel in which it occurs, since different rates of flow are found in different vessels[7]. Differences are found in this respect between veins and arteries, with venous thrombi tending to resemble the blood clot formed in the test-tube (the red thrombus), while arterial thrombi are more usually white due to a deposition of platelets. If the thrombus separates from the vessel wall, as commonly occurs in the heart and venous system, it can break free as an embolus (or plug).

Factors promoting thrombosis are described by Virchow's triad: (1) slowing of the blood stream; (2) changes in the vessel wall; (3) changes in the blood itself. These are summarized in the formula given above, $V.C/F = K$. Experimentally, thrombosis is not produced by one of these changes alone and it is probable that thrombosis is produced clinically by several of these changes occurring simultaneously—that is, *multiple causation.*

Thrombosis is a condition occurring in the cardiovascular system related to the normal, physiological function of haemostasis. Clinically, thrombosis is thought of as those situations where formation of thrombi has proceeded to the point where, by interfering with the circulation to an organ or part, there is interference with function and therefore symptoms result. The thrombus may produce these effects *in situ* or as an embolus produces such effects elsewhere. Arteriosclerotic changes in the arterial wall are the most common cause of thrombi forming in the heart and arteries.

Clinical effects produced by thrombi and emboli are: (1) no serious effects, the silent thrombus or embolus; (2) oedema of a limb, venous thrombus and perivenous lymphangitis; (3) post-thrombotic ulceration; (4) gangrene of a limb (thrombosis of main artery or embolus from heart or aorta); (5) gangrene of bowel (thrombosis of mesenteric artery or vein, arterial embolism); (6) infarction of myocardium, retina, or cerebrum through arterial thrombosis or embolus (an infarct is an area of coagulation necrosis, the word being derived from infarcire–to stuff); (7) cardiovascular collapse—large pulmonary embolus and vasovagal reflex.

Effect of Anticoagulants on Thrombosis

The effect of anticoagulants on thrombosis and embolism needs to be examined at different levels. Thrombosis was defined above as situations where formation of thrombi has proceeded to the point where there is interference with function and resulting clinical symptoms. To demonstrate that anticoagulants can prevent this process, lesions must be produced in vessels of macroscopic dimensions and examined by dissection in the living subject for the presence or absence of occlusions (i.e. studies on experimental animals). To determine what stages in the processes described in *Table 3.6* are affected by anticoagulants requires direct observation (microcinematography) of the processes taking place in blood vessels. To determine if the prevention of such occlusions will successfully prevent or change the clinical symptomatology resulting from the consequent physiological dysfunction, requires clinical investigations. It is important to distinguish the three aspects of the pharmacological problem and to appreciate that the limitations

of each approach means each approach can give the answer for only one aspect of the problem.

A number of investigators have demonstrated that anticoagulants prevent the formation of occlusive thrombi in vessels[5]. Anticoagulants have no effect on the initial platelet aggregation by collagen. They can inhibit viscous metamorphosis only in excessively large doses, and do not inhibit formation of microthrombi[257,269,270]. The action of anticoagulants is limited to inhibiting formation of larger thrombi held together by fibrin[271].

Clinical assessment of the effectiveness of heparin in preventing thrombosis and blood coagulation is a problem in operational research. It is indicated above that the largest amount of heparin is used today in the field of cardiovascular surgery and diagnosis. Direct assessment was made as these techniques were developed on experimental animals, where it was found that anticoagulants were an essential part of the technique. It is not practical or possible to carry out a clinical comparison of the results of using and not using heparin.

In the treatment of thrombosis and embolism after operations and deliveries, a number of clinical studies[5,7] have established the effectiveness of heparin and other anticoagulants in reducing the incidence of recurrence of thrombosis and embolism and in reducing the clinical sequelae of thrombosis—period of confinement to bed, hospital stay, convalescent period, handicap for normal living including such chronic symptoms as pain and swelling of leg after exercise, severe oedema, chronic induration and ulceration. This is the therapeutic role of heparin, with other anticoagulants, in the treatment of thrombosis and embolism already diagnosed in the patient. Other studies[272,273] have established the even greater effectiveness of heparin and other anticoagulants as a prophylactic measure in preventing thrombosis and embolism. For the general use of anticoagulants as a prophylactic measure after operations and delivery, there must be balanced the inconvenience of the anticoagulant treatment against the relatively low incidence of thrombosis and embolism. For this reason, prophylactic anticoagulant therapy is not widely used, but instead the heparin is given as soon as signs appear of impending thrombosis and embolism, such as changes in pulse, filling and hardness of individual superficial vessels, local changes in skin colour, skin temperature, oedema, unexplained rise in body temperature, Homan's sign, and observation by oscillography and phlebography. Anticoagulant therapy together with other measures has greatly reduced the incidence of recurrent thrombosis in these conditions[273]. While the incidence of thromboembolism after operation and delivery is relatively low, this is not to say it is not important. With the control of infections, thromboembolism has become one of the major problems accounting for one-third of the fatalities in obstetrical cases. The use of heparin and anticoagulants for control of thromboembolism is so well established that there is little justification in attempting assessments of its efficacy.

Recent clinical studies on the use of anticoagulants have indicated their use in cardiac infarction. This is a more difficult test for anticoagulants, since these drugs cannot change the damage to the heart muscle in the area of the infarct. Recent studies[4,5,8] agree that anticoagulant therapy is significantly beneficial to the survivors of severe coronary infarction when given up to one

year after an attack. Atherosclerosis is the common precursor of the thromboembolic diseases. A satisfactory treatment to arrest this ageing process in blood vessel walls would do much to reduce thromboembolism. Heparin and heparinoids are used for this on an experimental basis.

CHEMICAL STRUCTURE AND BIOLOGICAL ACTIVITY

Heparinoids and Mucopolysaccharides as Complexes, Clathrates, Ion-Exchange Compounds

Having considered the complexes which heparin (and heparinoids and mucopolysaccharides) form, we can ask the question whether such complexes are present in the materials as prepared. It is self-evident that such complexes must be present and this immediately explains why heparin usually occurs as a multi-component system.

We must consider the problem of complex formation from the standpoint of inorganic (and simple organic) anions and cations and from the standpoint of polysaccharide components of the system. Wilander[275] found that heparin gives the Hammarsten effect—the freezing point depression is much less than should be given by the sodium ion present. Higginbotham and Dougherty[276] first suggested that heparin functions as an ion-exchanger and while this was based on circumstantial biological evidence, recent physical studies have completely established the soundness of this speculation. Salminen and Luomanmaki[277] calculated from the distribution of the same ions between a heparin solution and its ultrafiltrate, that the activities of Na^+ and K^+ are markedly decreased by heparin and the potassium ion is bound by heparin in preference to sodium ion. Dunstone[278] found the reactions between acid mucopolysaccharides and various cations can be treated as ion-exchange reactions. There are differences in the order of increasing cation affinity for the different mucopolysaccharides. The order for chondroitin sulphate A is K, Na, Mg, Ca, Sr, Ba; for chondroitin sulphate B the order is K, Na, Mg=Ca=Sr=Ba; for heparin it is Na, K, Mg, Sr, Ba, Ca; and for heparin monosulphate it is Na, Mg, Ca=Sr, Ba. The differences in the order of affinity are attributed to differences in the structures of the various acid mucopolysaccharides. Counterions neutralizing electric charges are distributed throughout the ionic atmosphere with a fraction so close to the polymer skeleton as to be 'bound', that is a relatively mobile monolayer. The data indicate that heparin probably has a greater fraction of bound univalent cations and also indicate that the functional volume is relatively small. The concentration of cations in the polyelectrolyte phase is much greater than in the outside solution. Donnan equilibrium data indicate the co-ions are excluded, but if small the co-ions penetrate.

'Sodium salt' has a special connotation in describing these substances. The almost universal practice in analyses of heparin preparations is to burn a sample moistened with sulphuric acid in oxygen, weigh the ash, and calculate from the sulphated ash the equivalent sodium. This is evidently a questionable practice. The metal cations bound must be identified. As heparin and heparinoids complex with ions, there is interference with various colour and precipitation reactions for ions unless the heparin sample is first subjected to combustion. Flame analyses[279] of Boots and Evans heparin for

calcium, and neutron activation for strontium and barium, gave, on five batches, values for calcium of 300–2930, for strontium of 9–92, and for barium of 2·5–12 parts per million. Ammonium carbonate or hydroxide are used universally with heparin to give 'ash-free' preparations but when converted to a metal salt all the ammonium may not be replaced by the metallic cation, and nitrogen figures must be carefully analysed to distinguish amino and ammonia nitrogen.

The most common anions present are sulphate, phosphate, acetate and chloride. Helbert and Marini[42] determined titratable acid groups on a heparin from porcine intestinal mucosa, by hydrolysis with Dowex 50 (H^+) at 60°. Pretreatment with resin IRa-400 resulted in a drop of 6·6 (or 11 per cent) titratable acid groups per mole (or 11,900). The exchangeable acid groups were not dialysable and after removal could be replaced by adding sulphate. When tissue is extracted with potassium thiocyanate with phosphate buffer instead of the usual ammonium sulphate of Charles and Scott[280], the final product[281] has higher antithrombin potency and lower sulphur content than commercial heparin. The difference in sulphate is compensated for by phosphate, suggesting an exchange with inorganic salts in the initial extraction and representing binding sites made available when the protein-heparin bond is broken[282,283]. Because of complexing of inorganic sulphate in non-dialysable, exchangeable form, radioactive labelling presents special problems with heparin. Acetate is undoubtedly responsible for many of the acetyl values given in the literature. From infra-red spectroscopic evidence, the different polar groups of these polymers bind water vapour simultaneously according to steric accessibilities[284].

Oligosaccharides and polysaccharides are similarly bound to heparin, which usually contains about 10 per cent of other polysaccharide material. This can be distinguished by hydrolysis with hyaluronidase, or by giving a positive reaction with anthrone. Complexing with organic bases has been described in a previous section. Studies with these may throw light on the chemical structure involved in activity. An electrophoretic study of various commercial heparin samples having biological activity varying from 5–175 units/mg showed that they all contained identical components and distributions[44]. The streptomycin complex showed an interesting correlation with bioactivity. The data for the streptomycin complex is given as a function of sample activity, animal source and degradation. There appeared to be a relation between the biological activity and chemical structure in terms of matching of charges in the streptomycin complex.

The metachromatic reaction of heparin and heparinoids with dyes is an important key to the riddle of their chemical nature. A similar reaction is the starch-iodine reaction. This is due to the fixation of the iodine atoms in the starch micelle in an orderly array and is a well-known example of a clathrate, in which atoms or molecules are held in channels of larger molecules by strong, non-polar forces—hydrogen bond and London forces. It is the possession of a similar structure but with highly polar groups ($NH \cdot SO_4$, $O \cdot SO_3$, COO^-) in close proximity, which endows the mucopolysaccharides and heparinoids with their highly specific, characteristic properties.

Most preparations can be resolved into a number of fractions with paper chromatography, electrophoresis and fractional precipitation with acetone,

alcohol and dioxan or with anion-exchange resins or ECTEOLA. There can be as many as five different fractions. The question arises how many of these fractions are present in the original material and how many are produced by the manipulation.

We now have the information which indicates the basic problems in achieving a sample of heparin, which can be considered a single molecular species and therefore one for which a molecular structure can be constructed. Heparin can be changed from one 'salt' to another, e.g. barium to ammonium, by double decomposition. However, such double decomposition is not usually complete. Some sites of ion binding are less accessible than others. Some sites are so inaccessible that inorganic ions used in the initial extraction remain bound through the many chemical manipulations involved in purifying the compound. Organic anions and cations can be similarly bound to such sites, and if a sufficiently long chain, can result in the total molecule having a different molecular weight or total net charge. As a result, it is possible to obtain a series of components differing in these properties, and to continue to produce these as many times as one wishes to do so. For example, it is possible to elute the main component from a paper chromatogram and then rechromatograph to see as many multiple components as before. Laurent[286] chromatographed Vitrum heparin complexed with cetylpyridimine chloride on ECTEOLA columns and eluted at different ionic strengths to give four fractions with molecular weights of 11,800, 9,700, 8,400, 7,600, compared with 8,000, for the original Vitrum heparin. There was a corresponding decrease in nitrogen, hexosamine and sulphur values but a corresponding increase in the amount of ash in each sample. Hence, while one can draw correlations with the change in sulphur content, the changes in the ash content suggest the various correlations are simply due to increasing dilution with an inorganic component which influences the specific volume and apparent molecular weight.

Relation of Biological Properties to Chemical Composition

In attempting to relate the diverse biological properties to the chemical composition of this varied group of agents, experience has shown that the biological property in question can be due or related to any one of the following components or properties of the material being tested.

The preservative

Commercial heparin and heparinoid solutions commonly have phenol or tricresol added as a preservative. These are potent stimulants of isolated smooth muscle and pharmacological actions of heparin have been recorded due to the presence of these substances[163]. Pharmacologically active agents have been reported developing from the reaction of phenol with material in heparin[287].

The presence of accompanying protein, polypeptide or peptides

It is very difficult to remove the last traces of such material from heparin, yet this can be pharmacologically active. The histamine found in heparin after acid hydrolysis, as in the determination of normal blood histamine, probably arises from such peptides. Heparinoids prepared from natural

polysaccharides can similarly contain these. The antimitotic activity obtained from heparin by dialysis[288] had an ultra-violet absorption spectrum and was most probably a peptide.

The presence of accompanying polysaccharides

Heparinoids usually contain some of the original polysaccharide, while commercial heparin, as shown by the presence of anthrone-reducing substance, also usually contains such material. The elevation of body temperature, leucocytosis and thrombocytosis associated with pyrogens, can be due to the presence of extremely minute amounts of certain polysaccharides. One should suspect reactions of this nature as being due to traces of neutral polysaccharides.

The presence of low molecular weight substances of unknown nature

About 20 per cent by weight of heparin can be removed by prolonged dialysis of the heparinic acid[289].

The acid polysaccharide groups

Such activities should be markedly pH dependent, but are also related to the structure and orientation of the polysaccharide structure with resulting orientation and spatial relationship of the acid groups. In this regard, there should be a marked separation on the pH scale for effects of carboxyl groups and sulphate groups. The latter is responsible for the precipitation with benzidine and other complex bases in acid solution. There is a further difference in dissociation and possible biological activities between sulphate attached to nitrogen (the sulphoamino group) and the ester sulphate attached to carbon. Clearing factor activity is dependent on *O*-sulphate, not *N*-sulphate[290]; anticoagulant activity is dependent on sulphoamino[291].

Other linkages of groups

The distinction to be made between free and bound heparin probably relates to linkages with amino acids which are not salt linkages, and similar to those discussed for mucopolysaccharides. The involvement of zinc in the linkage of histamine to heparin in the mast cell[347] indicates the possibility of various tertiary systems being important in biological activity, as originally proposed by A. Fischer.

Molecular weight

Many investigators have attributed an important role to the molecular weight in determining biological activity in this group of compounds. Where it is possible to prepare a series of compounds from the same starting material, and with the same sulphate content as has been done with the dextran sulphates[293], a relationship can be established between molecular weight and biological activity, especially for the LD_{50}.

The combined effect of the major component and the considerable activity of a trace material present

While all the heparins, many derived heparins and many heparinoids are antilipaemic, polysaccharide fractions from a gram-negative proteus show

antilipaemic activity of the order of 1,000 times that of heparin[294]. This means it is possible (even if not probable) that a contaminant like the proteus polysaccharide is responsible for activity.

The ability of the main component to provide a clathrate or association complex

This is certainly the basis of the metachromatic reaction with dyes, and the ion-exchange properties. How many of the specific biological reactions of these compounds is due to this property requires extensive examination.

Coagulation Inhibition and Lipaemia Clearing After Intravenous Injection of Heparin and Heparinoids

The major use and interest in heparinoids at present is due to the fact they can be injected into the circulation to produce these two effects. In *Figure 3.3* are

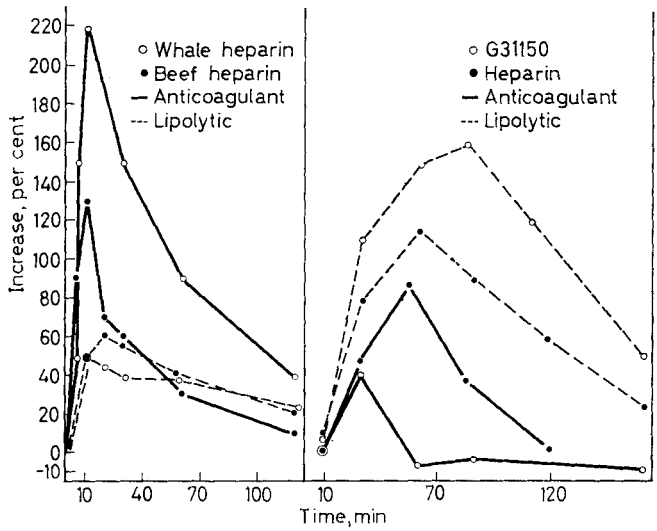


Figure 3.3. Effect of intravenous heparins and heparinoid on coagulation and lipolytic activity of blood *in vitro*[285, 295].
Values plotted as mean per cent increase over blood value before injection. Left-hand side, 1 mg/kg of whale heparin or beef heparin in each of 10 dogs. Clotting time by 4-tube Lee and White method. Lipolytic activity by 2 hour decrease in turbidity of sesame oil suspension. Right-hand side, 50 mg (0·8 mg/kg) of heparin in 6 humans or heparinoid G31150 in 5 humans. Clotting time as antithrombin time with normal value of 10 seconds. Lipolytic activity by 1 hour increase in free fatty acids with Ediol emulsion

shown typical curves taken from the literature. It can be seen that the response in all cases reaches a peak and then rapidly disappears, so that it has largely disappeared in two hours. It should be noted that the effects are not observed as soon as the heparin is mixed with the blood in the circulation. This is as true for the effect on coagulation as for the effect on blood lipaemia.

When the responses are compared for two heparins and the heparinoids, it can be seen that the two heparins gave the same antilipaemic response, while the whale heparin gives twice the anticoagulant response of beef heparin. The response with the heparinoid is slower but greater and more prolonged for antilipaemic activity than heparin, while the reverse was the

case for anticoagulant activity. These differences are generally found between heparin and heparinoids.

The response recorded varies markedly with the test used. Favre-Gilly[296] compared the response to 5,000 units of heparin in patients, as measured by the clotting time, thrombin time and Quick prothrombin time. Quite different responses were obtained. Thus, while one patient showed the textbook response of a marked increase in clotting time, slight increase in thrombin time, no change in prothrombin time, another patient showed almost the opposite, i.e. a slight increase in clotting time, a definite increase in prothrombin time and a marked increase in thrombin time. Similar differences are seen for the different tests used in following antilipaemic activity—clearing activity in plasma, free fatty acids of plasma, plasma cholesterol, electrophoretic distribution of serum lipoproteins, and esterase activity on prepared substrate.

Increasing the amount of heparin injected correspondingly increases the maximum response up to a limit. For the effect on coagulation, the limit is set by the amount of heparin cofactor present in the plasma; for the effect on lipaemia, this is set by the amount of enzyme[5] available for immediate mobilization. Further, each of these systems competes with the other for heparin[297]. Both of these limiting factors can be decreased by repeated injection of heparin or heparinoid.

In comparing the relative activity of heparins and heparinoids *in vivo*, the matching of response curves, as has been done in *Figure 3.3* is of limited value. What is required is a single value to represent the response. Two parameters define the response—the maximum value and the time to return to normal, but these values involve the largest experimental error in their determination. What is required is a value to express the total response calculated from all the determined experimental values. Millar, Jaques and Henriet[298] have described a procedure for this for the indirect anticoagulants. They calculated the area under the response curve, using the value before anticoagulation as the base line. The same procedure can be used for heparin and heparinoids. In calculating the area for coagulation tests, an adjustment must be made for the fact that there is not a linear relation between the values of the test (the various forms of coagulation time) and the concentration component changed, since this means that without an adjustment, the area value obtained will be influenced to too great a degree by large changes in the maximum value, and changes in the duration of the effect will not be seen. Millar, Jaques and Henriet have used logarithmic values. *Table 3.7* shows the values calculated in this way from the literature for the clotting time response in patients to heparin and to a heparinoid (Paritol). The area of the response curve is proportional to the dosage of heparin and of heparinoid.

Payne and Baker[301] tested a series of sulphated dextrans *in vitro* and *in vivo*. Their data have been recalculated and are shown in *Table 3.8*. *In vitro*, the anticoagulant activity in citrated plasma was from 0·09 to 20 per cent of that of heparin. For different preparations the value varied according to whether the end-point was the maximum amount which allowed clotting, or the amount for 50 per cent clotting or the minimum amount to prevent clotting completely. In contrast to the low anticoagulant potency *in vitro* are the results obtained *in vivo*. When the total response measured by the Lee and

Table 3.7. Relation between dose of heparinoid in man and area under the curve for the clotting time response

Drug	*Dose* mg	*Relative area* cm^2	*Area/Dose*
Heparin[300]	25	17·3	0·69
	50	34·9	0·69
	75	63·4	0·84
	100	84·5	0·84
	150	96·8	0·64
		Average =	0·74
	mg/kg		
*Paritol[299]	2	16·9	8·43
	4	25·0	6·26
	6	48·4	8·07
	8	104·0	13·00
		Average =	8·94

* Alginic acid sulphuric acid ester.

White clotting time was used as the parameter, similar responses were obtained for most of the samples. This suggests that the anticoagulant activity of heparinoids should be reassessed on the basis of quantitative *in vivo* comparisons.

Table 3.8. Comparison of the activity *in vitro* and *in vivo* of a series of sulphated dextrans, expressed as the percentage of the activity of heparin in the same tests[301]

%*S*	*Approx. mol. wt.*	*Anticoagulant activity*			*Toxicity* % *from* LD_{50}
		In vitro %	*In vivo Peak* %	*In vivo Area* %	
18·1	9,000	9	106	100	20
14·2	25,000	18	64	115	x
17·1	60,000	5·8	42	40	270
17·8	60,000	14	53	90	386
15·2	60,000	8	22	35	102
14·9	60,000	9	92	90	158
13·0	60,000	10	74	100	114
12·0	60,000	10	118	120	108
10·2	60,000	10	44	105	100
18·6	60,000	18	76	155	?
13·6	465,000	9	183	100	275

Anticoagulant activity *in vitro* calculated from the number of micrograms of dextran sulphate and heparin required to give 50 per cent inhibition of coagulation of 1 ml. dog plasma. Anticoagulant activity *in vivo* calculated from Lee and White clotting times at 0, 1, 2, 3 hours after intravenous injection in rabbits of 3 mg/kg. Peak = peak clotting times. Area = area under the curve with formula of Millar, Jaques and Henriet suitably modified. Values for heparin were: activity *in vitro*, 3·5 μg; *in vivo*, peak = 100 (92–108) minutes; area = 0·20; LD_{50} = 220 ± 42 mg/kg.

The failure of *in vitro* tests to reflect what may be the effect on coagulation *in vivo* can be due to many factors. Those heparinoids which cause clumping of platelets in the circulation can give an apparent enhanced *in vivo* response because of the increased sensitivity of the blood to the anticoagulant in a clotting time measurement. Release of endogenous heparin is also a possibility discussed on page 187. These must be distinguished from a true *in vivo* anticoagulant effect. A possible reason for failure of heparins and heparinoids to reveal their true anticoagulant activity *in vitro*, as pointed out to us years ago by Dr. T. Astrup in discussing the effect of acid and alkali on heparin, is that many workers fail to appreciate the extreme pH dependence of the anticoagulant action of heparin. Failure to check and adjust the pH and salt concentration of heparin solutions before assay can give erroneous results *in vitro*, although this will be a lesser problem *in vivo*. It is interesting that in a study[302] where hydrolysed heparin, and mucopolysaccharides were tested *in vivo* in lipaemic dogs for anticoagulant and antilipaemic activity, heparin subjected to 15 minutes heating at 100°C in 0·3 N NaOH gave clotting times and free fatty acid values identical to heparin, while heating in 6N NaOH gave values similar to chondroitin sulphate B, although there was no change in the clotting time or lipaemia with hyaluronic acid, chondroitin sulphate A or five seaweed extracts.

The anticoagulant activity of heparin can be abolished without affecting antilipaemic activity. *N*-(2,4-Dimethylbenzoyl)*N*-desulphoheparin with no anticoagulant activity is equivalent to heparin in the clarification of postprandial hyperlipaemia, experimental chronic hypercholemia and lipid emulsions *in vitro* by post-injection plasma. With *N*-formylchitosanpolysulphuric acid (Ro I-8307) in the rabbit[303], clearing factor activity increases with increasing dose so that the optimal dose is over 8 mg/kg, while heparin reaches a plateau at 1 mg/kg. In man the optimal dose is 0·25 mg/kg for heparin and 0·5 mg/kg for Ro I-8307. 'The action threshold for activation of clearing factor for both is 10 times lower in man than in the rabbit. The Howell time and antithrombin time are not inhibited by doses optimal for clearing factor.'

It is a remarkable fact that after 30 years of intensive effort in the synthesis of various heparinoid substitutes for heparin, it has not been possible to prepare compounds with greater anticoagulant activity than heparin, although compounds have been obtained with greater activity than heparin with respect to other biological properties. This suggests that this particular property of heparin is related to some feature of chemical structure not yet identified and which is additional to the carbohydrate skeleton, *O*-sulphate, or sulphoamino groups. The alternative suggested is that this situation is due to the use of inadequate methods of testing (*in vitro* tests).

Assay Procedures for Heparinoids

The assay of heparin has been discussed in detail by Jaques and Bell[304] and been recently given in a book by Tocantins and Kazal[305]. The possible properties of these compounds which have been used for assay, given in order of descending interest from the standpoint of pharmacology, are: (1) prevention of thrombosis; (2) anticoagulant activity on fresh whole blood; (3) meta-

chromatic activity; (4) inhibitor activity on coagulation factors and fractions; (5) inhibitor activity in different coagulation tests; (6) reaction with proteins or bases (precipitation, inhibition of enzymes); (7) physico-chemical properties such as electrophoretic mobility.

To the first point should be added antisteroid (cortisone, aldosterone), anti-inflammatory, anti-allergic, anticomplement and antihypertensive. Possibly a nomenclature is needed that distinguishes the various biological and therapeutic properties of heparin and heparinoids, as well as adequate standards for: heparin–anticoagulant, heparin–CF, heparin–anti-aldosterone, heparin–anti-inflammatory and heparin–antithrombosis.

The W.H.O. International Standard for heparin is a standard preparation, with 1 mg containing (by definition) 130 I.U. of heparin. However, the fact that a series of commercial heparin preparations (presumably all from beef tissue) gave similar values by several assay procedures (or a similar ratio compared with the International Standard) is no guarantee that this will be the case when heparinoids and heparin of different species of origin are tested, or when other biological parameters are investigated. In the international collaborative study of dextran sulphate, an international unit for dextran sulphate was defined as 0·04 mg of preparation set up for this purpose. What is needed are standard procedures using the international reference standard which are meaningful for the clinical use of the drug. This should be provided by the pharmacopoeias. It has been recently pointed out[306] that the pharmacopoeia descriptions of heparin and heparinoids are completely inadequate in defining and standardizing these drugs. The potency requirement in the U.S.P. monograph does not permit any real assessment of the biological efficiency *in vivo* and does not distinguish from chondroitin sulphuric acid; the colour requirement is not significant; the barium test is an outmoded remnant of early work; the nitrogen requirement is non-specific; the protein precipitation is inadequate and insensitive and does not test for peptides which are the most likely contamination. This is becoming a rather important practical problem from the standpoint of standardization of these drugs for clinical purposes. It is to be hoped that when the monographs are rewritten, potency limits will be in terms of clinical use—*in vivo* tests of antithrombotic, antilipaemic, or anticoagulant activities.

For pharmacological research on these compounds, in view of their extremely high activity in many systems, most tests are markedly sensitive and minute amounts of active substance are required. It is important to realize that there are two distinct problems, identification and measurement. There is a limitless number of systems available for measurement but there is no property, even the anticoagulant activity of heparin, which is not shared with many other substances. Heparin is only distinguished in terms of its very high specific activity, which means identification requires isolation of the compound, and measurement and observation of characteristic chemical and biological properties. Heparinoids are given in small doses and heparin itself is a trace substance in the body. This is a serious limitation to the reliability of much work on heparin. As with other highly active pharmacological agents, estimation of the substance by two or three different tests greatly enhances identification, for if the ratio of activities by the respective tests proves to be the same as for the reference solution, the reliability of the

estimation is increased. Important in this are the contaminants present, since different substances interfere in the different tests.

ABSORPTION, DISTRIBUTION AND METABOLISM

Heparin and heparinoids are absorbed in only trace amounts when given in large doses orally[307–309]. EDTA increases the absorption[310]. Subcutaneous and intramuscular injection of various depot heparin preparations have not been found very satisfactory. They fail to give satisfactory blood levels and they increase sensitization. With subcutaneous injection, the ability of heparin to become fixed to protein becomes a factor modifying absorption. One of the basic difficulties in deciding on the value of subcutaneous or intramuscular injections of heparin is that there has been no quantitative comparison made of the blood levels of heparin administered in these two ways, to determine how much appears in the circulation in active form. It is possible that much heparin never reaches the circulation. This is to be expected when it is remembered that heparin released by mast cells does not reach the circulation. Depository forms of heparin have been replaced by the use of concentrated heparin solutions (40 gm per cent), so that volumes of 0·2 to 0·4 ml. can be injected into subcutaneous fat tissue or intramuscularly.

From the figures of Eiber and Danishefsky[311], after the injection of ^{35}S-heparin into dogs at a level of 2–3 mg/kg, the heparin seems to be about evenly distributed in the blood between white cells and plasma, and in the plasma it is chiefly associated with Cohn fractions *I* and *III*. The attaining of equilibrium between heparin and blood constituents, as judged by the maximum clotting time reached after intravenous injection is a relatively slow process, requiring 15 minutes for heparin[312] and even longer for heparinoids (see *Figure 3.3*). Heparin is slowly distributed through the total extracellular fluid compartment[313]. Judging by experience with the large doses of heparin used in operations for the artificial heart, temporary take-up and storage of heparin (in the extracellular compartment?) may take place. In dogs, the intestine is the only tissue found with an increased amount of heparin after injection of large amounts of heparin.

Samuels and Webster[314] and later workers have drawn attention to the fact that uptake of heparin can be shown in the cement lines of vascular endothelium in the larger veins where endothelium is most easily studied. Electron micrographs show the cement lines to be the appearance of endothelial cell surfaces[315], so this may simply represent passage of heparin between these cells. The considerable amount of heparin deposited in the same way on the endothelial cells when injected intravenously, has a marked effect on the electrical potential of the cells[316]. Large amounts of heparin have been used in such experiments, so the heparin will be free and available for binding. It is important to know whether this occurs with bound heparin.

When heparin is administered intravenously, the material disappears from the blood very rapidly[312]. The relation between blood levels, rate of disappearance of heparin from the circulation and urinary excretion indicate one of the valuable pharmacological properties of heparin. It is cumulative only at low blood concentrations. At blood concentrations which give measurable clotting times, the rate of removal is dependent on the concentra-

tion in the blood but appears independent of blood level at higher concentrations in blood[270]. The point of change appears to be at a blood concentration of 2 units/ml. Below this level, a straight line relationship is obtained by plotting logarithmic clotting times against time which indicates a direct relationship between the rate of disappearance and concentration[312]. Olsson, Lagerren and Ek[317] have recently shown that in dogs for single doses between 200–800 units/kg elimination is exponential but with humans, this was true only for doses of 100 units/kg and with higher doses the elimination was only approximately exponential.

A further important point about the metabolism of heparin is made when heparins of different sources are injected *in vivo*[318]. Doses of 2·6 mg of beef heparin, 13·0 mg of sheep heparin, 8·6 mg of pig heparin, and 1·3 mg of dog heparin on intravenous injection gave the same pattern of change in the coagulation time. As 1 mg of each of these heparins contains an identical amount of sulphur and has identical metachromatic activity, this means that *physiological inactivation proceeds in terms of the anticoagulant activity* and *not of metachromatic activity* (or of those activities which are partly related to it, such as that used in the U.S.P. assay). It is usual to associate metachromatic activity with polymer size and degree of sulphation. This suggests that some other chemical property or grouping is responsible for the unique anticoagulant activity of the heparins.

Studies on the excretion products in urine after injection of heparin have demonstrated that the excretion products depend on dosage. With small doses to give concentrations of heparin in the blood of the order of 1 unit/ml. and using ^{35}S, it is possible to show that the sulphate part of 70 per cent of the injected dose is excreted as inorganic sulphate[319]. Therefore the injected heparin has been subjected to desulphation, presumably by a sulphatase. With increasing dosage, an increasing amount of sulphate appears in the form of mucopolysaccharide. This material has been identified for much longer, starting with the work of Astrup[320] and Jaques, Napke and Levy[321]. The mucopolysaccharide has the metachromatic activity of α-heparin but has largely lost its anticoagulant activity and is termed uroheparin. It shows slower electrophoretic migration than heparin. The renal threshold is 2·0 units/ml. of blood. With larger doses of heparin, unchanged heparin is found excreted in the urine. The renal threshold for this appears to be a level of 7·6 units/ml. of blood. Ordinary clinical single intravenous doses give mixed excretion of heparin and uroheparin, as well as inorganic sulphate, with a total excretion of about 20–30 per cent of the injected dose in the combined forms. The conversion of heparin to uroheparin is due to an enzyme heparinase[322] which has been extracted from rabbit liver. The desulphation enzyme has not been identified, although chemical studies[329] have shown that desulphation is a necessary first step to rendering heparin mucopolysaccharide susceptible so that it can react with various chemical degrading agents. Evidently, after desulphation has occurred, the mucopolysaccharide will be degraded by mucopolysaccharidases. Bacterial enzymes have been obtained[324] which cause an increase in reducing groups and an increase in the free amino groups and an increase in the periodate consumption, with hydrolysis of ester sulphate. Marbet and Winterstein[325] recovered about 50 per cent of the injected heparin in the form of uroheparin, with the dose used by them,

but observed that in the presence of protamine, no excretion occurred, the heparin presumably being all desulphated and degraded by mucopolysaccharidases. This indicates[326] that the change in type of excretion occurs when heparin is no longer all bound to plasma protein and indicates the difference in action of enzymes is related to the nature of the binding of the heparin. Where heparin is free in blood after injection, it is accessible to the action of heparinase and to direct excretion by the kidney. Where it is bound with coagulation protein or in another firmly bound protein-binding such as protamine, then it must be acted on by an enzyme causing desulphation of the heparin, which makes the heparin accessible to further degradation by mucopolysaccharidases.

Less is known about excretion patterns of heparinoids. Radioactive labelled sulphopolyglucin gives similar blood curves for radioactivity, metachromatic activity and whole blood clotting time.

TOXICITY

Haemorrhage, Hypersensitivity, Anaphylaxis

As explained on page 166, the development of serious haemorrhage requires multiple attacks on haemostasis and in this respect heparin is unique. Even though an anticoagulant and therefore a potential haemorrhagic agent, it prevents haemorrhage precipitated by stress and dicumarol, when given after these treatments[327]. In view of the many biological effects of heparin, it is remarkable that so many of its side effects are desirable, and not adverse as might be expected with such a potent drug. For this reason it is used clinically: this applies only in part to heparinoids.

'Inappropriate manifestations of heparin's therapeutic properties are avoidable by careful technique of administration. This will avoid local ecchymoses of minor or major proportions. Some of these are due to inappropriate dosages in susceptible patients with haemorrhage at distal and occasional critical sites such as the wall of the intestine. When heparin is administered to patients already on prolonged coumarin therapy, intractable bleeding may occur from factor *IX* suppression. Most frequently fatal, however, is the injudicious interruption of heparin therapy when bleeding presents. Permitting coagulation to return to normal is frequently associated with large emboli presumably shed by laminated clot appearing on injured endothelial surfaces.'[328]

As a result of the inhibitory effect of heparin on antibodies and anaphylaxis, allergic reactions are infrequent and true heparin sensitivity rare (one case of anaphylactic shock). Urticaria has been seen, induced by sensitivity to heparin's animal protein, and a histamine-like response at the injection site which gradually disappears if injections are maintained.

Toxicity of heparin and heparinoids has been examined in considerable detail from the standpoint of immediate and long-term toxicity. Initial and long-term toxicity of heparinoids is relatively slight with the initial material before sulphonation, but the introduction of sulphuric acid groups into the polysaccharide results in considerable toxicity. The effect of a series of polysaccharides and their sulphonated derivatives on leucocyte count, body temperature and E.S.R. in rabbits was that homopolar unbranched com-

pounds caused a fall of the leucocyte count and an increase in body temperature, increasing chain length increased the time of leucopenia, duration of leucopenia and raised the fever to a higher temperature, compounds with a polymer count over 200 caused an increase in E.S.R., while under 200 caused a decrease in E.S.R.[329] The effects on leucocyte count and body temperature are related more to neutral polysaccharides remaining in heparinoid preparations.

Immediate Collapse and Fibrinogen Precipitation

Immediate toxicity is a shock-like state or an anaphylactoid reaction and can be in general described as agglutination of platelets and precipitation of fibrinogen to give pulmonary emboli. Both these effects are closely related to the molecular weight or polymer size[330–333]. Histological findings suggest strongly that the pathological changes produced with dextran sulphate are caused primarily by the intravascular precipitates occluding capillaries[333].

Long-term Toxicity-collapse Syndrome

On long-term toxicity tests, many heparinoids such as sulphated chitin, laminarin sulphate, Elheparin, thrombocid[334] and sulphopolyglycin[258] prove fatal when given intravenously at 2–3 times the clinical dose. Cronheim[258] describes the syndrome in terms of the same sequence of toxic symptoms: loss of appetite, weight loss, severe diarrhoea containing grossly visible blood, and sudden death. Autopsy shows extensive haemorrhagic inflammatory processes, but without ulceration in the intestines, which, in the case of sulphopolyglucin, are restricted to the luminal layers and are characterized by venous capillary stasis. With some heparinoids, inflammatory and degenerative changes are also found in the liver and the kidneys. The animals tolerated the drugs without difficulty for a certain length of time, varying from a few days to 6 months, depending on the dose and route of administration. Suddenly, the above-mentioned sequence of toxic effects commenced. Death resulted invariably within a few days, but it could be averted by immediately discontinuing the drug and by repeated blood or plasma transfusions and special nursing care.

Even with heparinoids which do not produce serious clinical symptoms on long-term toxicity tests, various pathological changes are found in organs on histological examination. Frequently, accumulation of metachromatic material is found in the liver with storage in histiocytes which is different from mast cell phenomenon. Retention of heparinoids has been established by histological staining methods[335] and by the use of ^{35}S-labelled compounds[336]. Cronheim[258] described the long-term toxicity with the collapse syndrome; the collapse is not due to haemorrhage or blood loss, but is related to the accumulation of the heparinoid in the Kupffer cells of the liver and possibly in other organs. The release of the heparinoid from the reticuloendothelial system is extremely slow. The gross signs of toxicity and the characteristic pathological changes in the intestinal tract show some resemblance to endotoxin intoxication[337]. With the heparinoids retained in the Kupffer cells, Cronheim suggests that the detoxification mechanism for endotoxin is impaired, and that the animals die from an accumulation of endotoxin.

Alopecia

On clinical trials, a specific side effect has been found with all these compounds on long-term administration, namely alopecia[338]. Beller[339] reports that the incidence of loss of hair in 8–12 weeks on long-term therapy amounts to 70 per cent with heparin and 100 per cent with G31150. The alopecia is of varying degree of severity. It can result in complete temporary baldness or simply an increased rate of shedding of hair. It is completely reversible. When the administration of the heparinoid is stopped, the hair returns and with grey hair, the new hair may be coloured. The relationship of heparin and heparinoids to hair growth is not clear. It has been assumed that this may be a competition with the mucopolysaccharides involved in hair growth and formation of keratin. With the low concentration of heparin and heparinoid in the follicle, the effect is much more likely an expression of interference with hormone activity.

Bone Changes

A toxic effect on bone growth was observed with laminarin sulphate[340] but not with other heparinoids. The animals showed loss of weight, frail appearance, skeletal changes, the carpal joints became knobbly, and there was splaying of feet and kyphosis. The changes were greatest at the growing ends of long bones and the primary effect was a disturbance on the endochondral bone formation. This probably represents interference with chondroitin sulphate metabolism and emphasizes that the other effects of heparinoids are probably not related to chondroitin sulphate metabolism.

ENDOGENOUS HEPARIN AND HEPARINOIDS

The Mast Cells

The identification in 1937 of the staining properties of heparin with metachromatic dyes with the similar staining of the mast cells by Wilander[341] meant the satisfactory identification of the source and location of much of the heparin in the body. Recent descriptions of the mast cells are given by Bloom[342] and Smith[343]. Equally important was the identification of histamine as the other important pharmacologically active component of the mast cell by Riley, Shepherd and West[323]. This is further discussed in the book by Riley[344]. In general, there is a rough correlation between the distribution of heparin, histamine, hyaluronic acid and the mast cells.

The identification of the mast cells recognizes local accumulations of highly concentrated heparin; the chemical extraction requires large amounts of tissue. While a correlation between mast cell count and heparin content of tissues has not been established[3], the relationship between these two seems to be sufficiently close to indicate that all heparin is associated with mast cells. This does not prove that in ordinary tissue the mast cells contain the heparin and none is from the surrounding connective tissue matrix. Normal mouse and rat mast cells contain heparin and no other polysaccharides[75,345,346], but solid mast cell tumours of mice also contain chondroitin sulphate-like polysaccharides, as an ascites subline of the Furth tumour and dog mastocytomas gave nonsulphated polysaccharide material.

Histamine and heparin seem to be in tight combination in the mast cell,

yet the complex formed *in vitro* dissociates readily. Kerp and Steinhaüser[346] have shown that heparin and histamine are bound much more effectively when a ternary complex is formed with zinc and that there is a corresponding concentration of zinc in the mast cell granules released by compound 48/80, suggesting that this provides the necessary stability to the complex in the granules[347,348]. The presence of 5-HT in rat and mouse mast cells appears to be associated with a 'unique heparin', immobile on paper chromatography[32,349].

Considerable information has been obtained recently on the steps in the metabolic transformations to heparin, through studies on mouse mast cell tumours in slices and homogenates. It has been established that this takes place in the granule. ^{14}C-Glucose and ^{35}S-inorganic sulphate is converted by mouse mast cell tumour slices into mucopolysaccharide indistinguishable from heparin. It appears that the sulphate is incorporated into heparin precursors of low molecular weight probably phosphoadenosine phosphosulphate[350] and these are then incorporated into the heparin fraction. Higginbotham[351] showed that fibroblasts take up heparin as metachromatic granules and it is almost impossible to distinguish between heparin-forming mast cells and heparin-digesting fibroblasts by simple inspection of slides[352], but the pseudomastocyte granule in the heparinophage can be distinguished from the true mastocyte by the fact that it disappears under the action of heparinase. Velican and Velican[354] conclude that cells with the appearance of tissue mast cells represent different reticulohistiocyte elements, especially macrophages following excessive accumulation of polysaccharide under the action of certain irritant factors. An extreme form of this is seen when heparinoids and many other substances are given. Cells appear which are largely metachromatic but different and easily distinguished from mast cells.

According to West[356], the biological significance of the pharmacologic agents in the mast cells is related to the connective tissue. In the embryo, there appears to be a general metachromasia of tissues which finally results in metachromasia in the mast cell. Histologically, the mast cells appear to undergo cyclic changes so they alternately store and release substances to the ground substance mucopolysaccharide, i.e. hyaluronic acid. This is not for synthesis of ground substance, because chemically heparin and hyaluronic acid are different substances and there is increasing evidence that synthesis of both is from different units. Also, if mast cells are responsible for the production of ground substance, one would expect some indication of this in healing wounds, but mast cell counts[355] show that newly-produced ground substances and mast cells appear separately. Since minute amounts of heparin inhibit *in vitro* the depolymerizing action of hyaluronidase on ground substance, possibly mast cells act as hyaluronidase inhibitors in the evolution of granulation tissue. West supports Riley's view that mast cells releasing histamine prepare many more connective tissue cells to receive the consequent heparin and bradykinin releasing enzyme. This potent vasodilator is responsible for the increase in capillary permeability, accumulation of materials and pain in connective tissue in response to foreign materials, i.e. trephocytosis.

The injection of toxic agents such as compound 48/80 and Russel Viper Venom results in disruption of mast cells with shedding of granules. Higgin-

botham and Dougherty[14,276,351,357] have shown that heparin protects mice from these agents and that furthermore the difference in lethal effects of intravenous compared to subcutaneous injections can be attributed to the fixation of the noxious substances by the acid mucopolysaccharides released. This fixation is followed by ingestion of the complexes by fibroblasts. For this, Higginbotham has devised the term 'micellophagosis' (*Figure 3.4*).

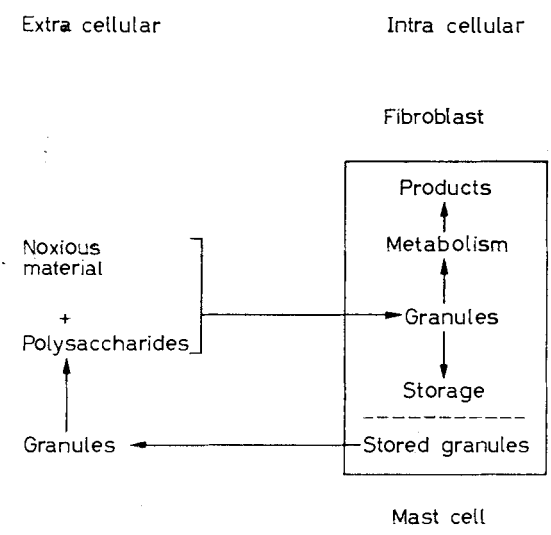


Figure 3.4. General scheme of 'micellophagosis' and detoxication[357]

Hyaluronic acid is ingested by fibroblasts but appears as vacuoles rather than discrete granules. Polysulphated hyaluronic acid and chondroitin sulphate A are taken up by fibroblasts in granular form but remain in nondigestive form in cytoplasm thirty days later. This is true of many foreign polysaccharides. Higginbotham suggests that the mast cell represents a type of mesenchymal cell which takes up mucopolysaccharides from the ground substance, storing the mucopolysaccharides cytoplasmically and showed[351] *in vitro* that compound 48/80, polymyxin B, neomycin, protamine, and clupeine can displace toluidine blue from heparin. Intraperitoneal injections of heparin into mice 30 minutes before the intravenous injection of otherwise lethal doses of these same agents markedly enhance tolerance. Less marked tolerance could be induced by chondroitin sulphate B but not by chondroitin A and hyaluronic acid. Sulphated derivatives of dextran, polygalacturonic acid, polymannuronic acid, levan, alginic acid, and cellulose were highly protective against the lethal doses of compound 48/80, polymyxin B and stilbamidine, whereas nonsulphated dextran was inactive and carboxymethylcellulose only weakly active.

Heparin and Heparinoids in Blood

There has been continuing interest in the possibility of heparin being present in blood ever since the first descriptions of the anticoagulant. In fact, it is almost universally assumed that it must be there. Surprisingly enough it has

been very difficult to demonstrate any significant amounts of heparin in normal blood. This is in contrast to the excessive amounts of heparin which can be demonstrated in the blood of dogs after peptone and anaphylactic shock, a subject under active study for 80 years (cf. Delezene[358]). The identification of the marked anticoagulant activity appearing in these conditions as due to release of heparin from the dog liver was completed with the quantitative isolation of heparin in crystalline form by Jaques and Waters[359] and the demonstration by Scroggie and Jaques[360] that it is possible to obtain equivalent amounts of histamine and heparin by antigen from the isolated perfused liver. The heparin is released into the lymph. Release of heparin does not occur with histamine shock or with haemorrhagic shock. No other conditions (except papain injections) have been found to release heparin. Accompanying liberation of histamine and heparin, there is some protein breakdown[361] and soya bean trypsin inhibitor has some inhibitory effect for release by peptone. This suggests that proteolysis may be a part of the factors in the liberation of histamine and heparin. In the absence of the liver, relatively little heparin is released by antigen in sensitized dogs and the amount of histamine released is only an insignificant fraction of the amount released from the liver[359,362]. Assuming the same degree of sensitization, the question arises as to whether other mast cells do not release these pharmacologically active agents. This may be due to the location of the mast cells. Mast cells are located in tissues, and there is a marked barrier to the immediate access of the cells and their products to the circulation, namely the thickness of the vessel wall, but in the liver due to the location of the hepatic lymph channels, the pharmacologically active agents released by the mast cells are immediately carried via the thoracic duct to the general circulation. In other organs, probably local fixation of these occurs in the connective tissue matrix so that practically none appear in lymphatics and thus in the general circulation.

Anticoagulant material found in blood of pathological cases has rarely been found to be related to heparin. A number of other anticoagulants have been described—proteins[363,364], sphingomyelin, sphingosine, inositol phosphatides, phosphatidyl serine, lipid-protein[365], profibrin, or other products of fibrinogen acting as anticoagulants[367,368].

The various methods developed for the determination of heparin in blood after its addition either *in vitro* or after injection, have been applied by many investigators to the question of whether there is any heparin present in normal blood—titration with protamine, toluidine blue[369], polybrene and 'Heparin tolerance' tests. Greenspan[370] isolated mucoproteins from dog, ox and human plasma and showed that these caused an increase in the Lee and White clotting time. They were neutralized by protamine in a ratio of 5·5 mucoprotein to 1 of protamine. All sera which had an increased protamine had increased mucoprotein levels: this is the 'heparinoid' of serum. Variations in protamine titration are accounted for by 1–2 mg of mucoprotein/ml. Hence the elevation of mucopolysaccharides in plasma and mucoproteins can be responsible for the titration values obtained and the large number of papers which refer to 'blood heparin' levels should be recognized as referring to blood 'heparinoid or mucopolysaccharide' levels.

However, a number of studies have identified heparin as a trace constituent of normal plasma[320,371,372]. The least concentration of free heparin which

can be demonstrated by anticoagulant activity is 1 μg/ml., while for clearing factor it is 0·1 μg/ml. As there is not an excess of anticoagulant activity in normal blood, the value obtained for normal human blood for heparin can be taken as an indication of the specificity of the method for heparin. Values of the order of 10 μg/ml. for normal blood plasma indicate that the method is measuring mucopolysaccharides such as chondroitin sulphate or mucoprotein. Values of the order of 1 μg/ml. for plasma indicate that this is probably a measurement of heparin, particularly if proteolytic digestion is included in the procedure so that the determination is of bound heparin rather than free heparin.

There are specific methods for heparin and these have yielded positive results for heparin in normal blood. The problem is complicated because a distinction must be made with heparin in normal blood between the white cells, platelets, bound heparin in plasma, and free heparin in plasma. Methods are now available for all of these. It can be shown that after injection, free heparin can be extracted from the blood and determined[366]. On the other hand, in contrast to this, the heparin normally present in plasma, not resulting from injections, is firmly bound and best estimated by the methods of Engelberg[373], based on the older studies of Jaques, Monkhouse and Stewart[374], Nilsson and Wenckert[371] and Astrup[320]. Engelberg gives a value of 1–2·4 μg/l. (or 0·1–0·24 units/ml.). This minute amount is probably not significant as far as blood coagulation is concerned, although it does appear to be significant in terms of serum cholesterol. The basophilic leucocytes in the blood usually contain chondroitin sulphate but they may contain heparin. In the method of Bassiouni[375], plasma and white cells are extracted separately and final identification is by measurements to determine free heparin and mucopolysaccharides in plasma and white cells simultaneously.

Snellman, Sylven and Julen[376] isolated the heparin polypeptide and showed that this material is a potent antithrombin on thrombin with purified fibrinogen, suggesting that heparin in the mast cells is in the active anticoagulant form. Electrophoresis shows that this native heparin forms a complex compound with thrombin and also with a lipoprotein molecule. They conclude the whole heparin complex is produced in the intergranular cytoplasm of the tissue mast cells.

Physiological Significance of Heparin

In order to establish the physiological significance of heparin, a series of paradoxes which have been presented in this review must be recalled. First, while heparin was introduced to medicine because of its anticoagulant properties in the circulation, it shows many more effects through mobilizing or suppressing the release of enzymes from tissues and hormones from endocrine glands. In addition to affecting the release of enzymes or hormones from cells, heparin can also affect the action of these agents at the target cell or organ. The problem is to show that heparin does appear in the circulation in response to changes where heparin secretion could be an appropriate response. This has been shown for lipaemia clearing. Lipaemia results in the appearance of clearing factor. All attempts to show increased heparin in the blood with hypercoagulability by testing the effects of coagulants and of thrombosis have been negative. Other systems, such as hyperaldosteronism

have not been evaluated from this standpoint. However, Kozewski and Vahabzadeh[377] have recently reported a case showing absence of heparin cofactor in plasma with resulting resistance to heparin; the patient had been hospitalized for recurrent thrombotic episodes. When the heparin cofactor level in the blood was restored to normal by a course of fever therapy, the patient had no more thrombotic episodes. This suggests that heparin has a physiological role in preventing thrombosis but the only evidence is this one single clinical case.

The difficulty in obtaining satisfactory evidence may be due to the fact that thrombosis is a local circulatory problem which will require release of heparin locally for control. Such amounts will not be apparent in gross biochemical tests either as an increase in plasma concentration or a decrease in concentration in tissue. It is probable that heparin will be like other autopharmacological agents (e.g. adrenalin, steroids, insulin) in that the amount of heparin in the general circulation at any one time is only a secondary reflection of secretion levels. More important is the determination of rate of urinary excretion of metabolites and still more important the determination of rate of secretion by the glandular tissue (mast cells) itself.

Crucial evidence regarding the physiological significance of body constituents has frequently been given by diseases which represent over production of the constituent. Systemic mast cell disease or urticaria pigmentosa is characterized by symptoms of histamine poisoning. Any bleeding that occurs is due to thrombocytopenia and the increased prothrombin time. There is no evidence of heparin in the blood[378]. The one possible example of clinical bleeding due to heparin in mast cells is that reported by Meneghini[379]. He found that in juvenile epistaxis, biopsies of the nasal mucosa demonstrated a marked accumulation of mast cells and that local medication with toluidine blue was helpful in controlling the haemorrhage. Here it is possible that there was local liberation of heparin. The increased pigment formation in urticaria pigmentosa may be a reflection of the action of heparin accelerating pigment formation. Only three cases of hyperheparinemia are recorded in the literature and only one of these has been fully documented[380]. This does not mean that the condition is necessarily rare. Like the Hageman and Panter traits, the condition is symptomless. The one documented case was discovered in a woman only when there was serious post-partum haemorrhage, although the condition was undoubtedly present from childhood. There was no evidence of increased numbers of mast cells.

It has been pointed out that the mast cells are outside the blood vessels and access by their secretions to the circulation is only via lymphatics. It is possible that heparin influencing the circulation might come from the vessel wall rather than from the mast cells. An anticoagulant mucopolysaccharide closely resembling heparin can be extracted from human aorta[381]. Accompanying this is an antiheparin factor[382] which appears to be highest in normal aortic walls and decreases with increasing arteriolar arteriosclerosis. However, this is possibly from mast cells located through the wall thickness and particularly in the adventitia. More direct evidence is that of Marin and White[383] who report that slices of aorta cause some loss of activity of thrombin solutions which is prevented by polybrene and that likewise slices cause loss of activity of serum in the thromboplastin generation test. Histologically

there is loss of metachromatic activity without any irregularity of endothelium appearing, so that this suggests a possible release from the vascular wall. Where does this heparin come from? The uptake of intravenous heparin on endothelium (at the cement lines) has already been described; it might be possible that heparin from mast cells reaching the circulation in the lymph may be deposited here. It would then change the electric charge and protect the endothelium from thrombus formation[316]. The recent discovery[384] of heparin bonding on colloidal graphite surfaces previously rinsed with a cationic surface-active agent giving a surface highly resistant to clot formation for plastic or silicone surfaces, is then not only of great practical importance in the construction of cardiovascular protheses, but may be a model of the physiological state of the endothelium.

The diversity of actions of administered heparin and the demonstration that some of these actions take place with endogenous heparin suggest that heparin exerts both local and general physiological effects. Contained as it is in the mast cells, it takes an active part in the local processes in connective tissue and in so doing is an important part of the local defence mechanisms of the body. On the other hand, if heparin enters the circulation it will produce general effects through modifying the electrokinetic charge of endothelium and the release and action of hormones and enzymes in the circulation. These can be summarized as growth and repair of connective tissue; preservation of blood flow (effects on blood coagulation and charge on endothelium); general regulating function in cells on protoplasmic clotting and ion exchange; control of fat metabolism (through release of lipoprotein lipase); natural defense mechanisms (detoxifying action, anti-stress).

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Examination of heparins by nuclear magnetic resonance by Jaques *et al.*[268] has shown equal amounts of three carbohydrate components in undegraded heparins. Dr. A. S. Perlin has identified the third component as iduronic acid.

4

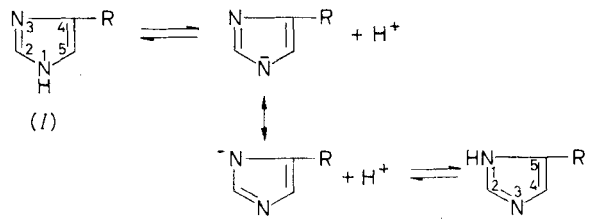
THE HISTIDINE DECARBOXYLASES

D. M. SHEPHERD AND D. MACKAY

INTRODUCTION

Nomenclature

IMIDAZOLE derivatives in which the nitrogen atoms of the imidazole ring are unsubstituted exist in the tautomeric forms (*I*) and (*II*) which are in equilibrium:

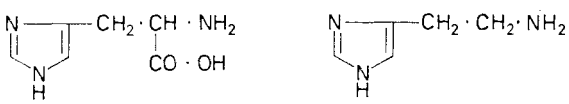


(*I*) (*II*)

The numbering of the ring atoms of imidazole begins at the imino-nitrogen atom and the other nitrogen atom receives the number 3. The tautomeric forms (*I*) and (*II*) are thus numbered as shown; hence C_4 and C_5 of the imidazole ring are equivalent. It follows that 4-methylimidazole (*I*, R = Me) and 5-methylimidazole (*II*, R = Me), for example, are identical, and it is customary to name this compound 4(5)-methylimidazole. Other monosubstituted imidazoles in which the substituent is attached to C_4 or C_5 are similarly named.

Histamine and Histidine

Histidine, β-4(5)-imidazolyl-α-aminopropionic acid (*III*), is one of the essential amino acids. From the pharmacological point of view, it is of particular importance as it is the immediate precursor of the highly active amine histamine, β-4(5) imidazolylethylamine (*IV*).



(*III*) (*IV*)

Histamine, which is widely distributed in nature, is formed by the decarboxylation of L-histidine by the enzyme histidine decarboxylase. Non-enzymic decarboxylation of histidine has also been observed[1–4] but the conditions under which this occurs render the reaction of little physiological interest.

In view of its extremely high biological activity and the fact that it appears to be implicated in a variety of pathological conditions in man, histamine has been studied much more extensively than the enzyme which gives rise to it. It is only recently that histidine decarboxylase has been subjected to detailed study. Various aspects of this work are now surveyed.

THE MEASUREMENT OF HISTIDINE DECARBOXYLASE ACTIVITY

The activity of histidine decarboxylase preparations can be measured by determining either of the decarboxylation products of histidine, i.e. histamine or carbon dioxide. Most of the methods are *in vitro* techniques using organ slices, minces or cell-free extracts, and they have the usual limitations of *in vitro* measurements[5,212,245]. *In vivo* methods have also been used, but these give information primarily about the decarboxylation of histidine in the body as a whole rather than about the distribution of the enzyme in specific organs.

In vitro *Measurements*

Measurement of carbon dioxide production

While the carbon dioxide produced by decarboxylation of histidine can be measured by the standard Warburg manometer technique, the utility of the method is severely limited by the very small degree to which decarboxylation occurs with most mammalian histidine decarboxylase preparations even at high substrate concentrations. In practice, only bacterial histidine decarboxylases have proved sufficiently active to be measured conveniently by the manometric method[6]. Small amounts of carbon dioxide can, however, be determined by the sensitive micro-diffusion technique of Conway[7], and this has been used successfully for measuring the activity of mammalian histidine decarboxylases[8].

The most sensitive method for measuring carbon dioxide production by histidine decarboxylase is that in which histidine labelled with carbon-14 in the carboxyl group is used as substrate. The $^{14}CO_2$ evolved is trapped in a suitable absorbent medium, and its radioactivity is then determined directly in a scintillation spectrometer. Several variations of this procedure have been described[9,10,11,242,256], and these compare favourably in speed and sensitivity with alternative isotopic methods in which the production of radioactive histamine from ring-^{14}C-labelled histidine is measured (see following section).

Measurement of histamine production

The histamine formed by the decarboxylation of histidine can be measured by the standard biological method using the atropinized ileum of the guinea-pig. This procedure, which has the advantage of simplicity and high specificity, is not unduly time-consuming when an automatic assay apparatus is used[12] and the necessary calculations are made by means of an electronic computer. A tissue blank must be carried out to determine the amount of endogenous histamine derived from the tissue during the preparation of the enzyme extract. An additional blank experiment can be included to allow for histamine present in, or formed by non-enzymic decarboxylation from, the

histidine[13,14]; this is particularly important when tissues of very low histidine decarboxylase activity are under investigation.

Alternatively, the histamine can be determined spectrophotofluorimetrically[15,16]. With this method, it is essential to remove histidine after the incubation, as the substrate would otherwise contribute to the final fluorescence.

These methods are not sufficiently sensitive to detect the small amounts of histamine produced by mammalian histidine decarboxylases when low substrate concentrations (<50 μg/ml. histidine) are used. The histidine concentration in the blood plasma of most of the animal species commonly studied in the laboratory is approximately 10 μg/ml.[17] Therefore *in vitro* measurements at substrate concentrations of this order assume particular physiological significance. The sensitivity of the above methods can be greatly increased if the histamine produced is concentrated, and freed from histidine, by means of a cation-exchange resin; in this way determinations have been made at histidine concentrations below 1 μg/ml.[16]

However, the use of radioactive techniques is to be preferred for measurements at these low substrate levels. The histidine used is labelled with ^{14}C in the 2-position of the imidazole ring, and the resulting radioactive histamine, diluted with non-radioactive carrier, is converted to a suitable crystalline derivative which is then purified and counted in the normal way. In the method as originally introduced[18,19] the histamine was crystallized as the picrate. More recently Schayer[20] has isolated the histamine as the dibenzenesulphonyl derivative (BSH), the activity being measured by the liquid scintillation technique. An alternative method[21] is to prepare the di-(*p*-iodobenzenesulphonyl) derivative (pipsylhistamine) the activity of which is then measured at infinite thickness in a gas-flow counter; this counting procedure has the disadvantage of being less efficient than the liquid scintillation method. Methods have also been described in which the radioactive histamine formed, instead of being converted to a derivative, is separated from the substrate by paper chromatography before counting[22,255].

Since only the radioactive histamine formed from ^{14}C-histidine is measured, the endogenous non-radioactive histamine in the tissue need not be taken into consideration, and so no tissue blank is necessary. The method is somewhat tedious, however, as complete separation of unreacted histidine from the histamine formed is essential; this involves either partition between suitable solvents, or separation on an ion-exchange resin. Moreover, the ^{14}C-histidine available commercially contains a small amount of ^{14}C-histamine, and it may also give rise to further traces of ^{14}C-histamine by nonenzymic decarboxylation during the incubation and the subsequent analytical procedure. As the histamine derived from these sources is sufficient to interfere in this highly sensitive method, a substrate blank must be carried out[23]. To reduce the activity of this blank to a minimum, the ^{14}C-histidine should be freed from ^{14}C-histamine by ion exchange before use. There is evidence[24] that where the blank is carried out in the presence of the enzyme, but with the addition of sufficient semicarbazide to prevent completely the occurrence of enzymic decarboxylation, the semicarbazide also inhibits non-enzymic decarboxylation. Since the histidine decarboxylase activity of the tissue is the difference between the blank and the uninhibited reaction, the resulting low blank would lead to a falsely high level of the enzyme being obtained. This

error would be most serious in tissues of low activity, and it could lead to activity being observed in tissues which actually contain no histidine decarboxylase whatsoever.

In vivo *Measurements*

As is the case for most enzyme activities measured *in vitro*, there is some doubt whether the histidine decarboxylase activities determined as above in various organs truly reflect the contribution of these organs to histidine decarboxylation in the intact animal. *In vivo* measurements give an overall picture of histidine decarboxylation in the living animal, but they can give little indication of the contribution made by individual organs. Moreover, the interpretation of such measurements is rendered difficult by bacterial decarboxylation of histidine in the gut, by metabolic destruction of histamine, and by the release of histamine from storage sites. Nevertheless, such measurements have provided much useful information, and they are particularly suited to the study of the effectiveness of histidine decarboxylase inhibitors in intact animals. As with *in vitro* methods the *in vivo* measurements can, in theory, be made either on the carbon dioxide or on the histamine formed in the decarboxylation.

Measurement of carbon dioxide production

The carbon dioxide evolved by the decarboxylation of histidine would normally be overshadowed by that produced in respiration, but this difficulty could be overcome by the use of histidine in which the carboxyl group was labelled with ^{14}C, the expired $^{14}CO_2$ being collected in a suitable medium for counting. Although this method does not appear to have been tried for histidine decarboxylase, it has been successfully applied to a study of the *in vivo* decarboxylation of other amino acids containing $^{14}COOH$[25,26].

Measurement of histamine production

Two methods can be used, both of which have been developed with particular reference to studies in the rat. Female rats are normally used as they excrete most of their urinary histamine in the free form; in males, much of the urinary histamine is in the form of the ring-*N*-methyl derivative which is difficult to assay. In the non-isotopic technique, the animals receive a standard semi-synthetic histamine-free diet; the 24-hour urinary excretion of free histamine, assayed biologically, then gives a measure of histamine formation *in vivo*[27,28]. Alternatively, if histidine labelled in the 2-position of the imidazole ring is injected, the excretion of the resulting ^{14}C-histamine in the urine may then be followed[29,30]. The use of these two methods in conjunction has been advocated[28], the biological method providing a preliminary screening test for alterations in histamine output, while the radioisotope procedure permits any changes to be analysed in greater detail. The radioisotope method has also been applied to perfusion experiments *in vivo*, ^{14}C-histidine being perfused through a suitably cannulated organ of an anaesthetized animal and the perfusate collected for histamine analysis[23,31]. As in the case of *in vitro* experiments, allowance must be made for traces of ^{14}C-histamine in the commercial histidine.

Of these procedures for measuring histidine decarboxylase activity *in vitro*

or *in vivo*, bioassay is quick and specific, but it is insufficiently sensitive with preparations of low enzyme activity or where very low substrate concentrations are used. The high specificity and sensitivity of the radioisotope method allow it to be used at substrate concentrations within what is usually regarded as the physiological range; however, the manipulations are frequently laborious and time-consuming and the cost per experiment is high, particularly when *in vivo* studies are made. Spectrophotofluorimetric assay occupies an intermediate position from the point of view of speed, but, being more liable to interference from other biological materials, it is somewhat less specific than the other methods[145,213,214,241].

THE SUBSTRATE SPECIFICITY OF HISTIDINE DECARBOXYLASES

For many years two main types of histidine decarboxylase were recognized, one of bacterial origin with its optimum activity at pH 4[32,33], the other, occurring in mammals, having its optimum activity in the range pH 8·0–9·5. The preparation of histamine from histidine under the influence of bacteria was first carried out by Ackermann[34]; shortly afterwards histidine decarboxylase was shown to be present in *B.coli*[35]. Histidine decarboxylase of mammalian origin was first detected in rabbit kidney[36]. Although the present survey is confined mainly to mammalian histidine decarboxylases, the bacterial enzyme cannot be entirely ignored, since its presence in the intestinal flora of many mammals must be taken into account, especially in the interpretation of *in vivo* experiments[37]. The properties of bacterial histidine decarboxyllase have been reviewed by Gale[6].

When the development of more sensitive techniques and the introduction of new inhibitors had made more detailed studies possible, it became clear that mammalian histidine decarboxylases could themselves be divided into two sub-classes, one having optimum activity in the range pH 6–7, and the other in the range pH 8·0–9·5[20,38]. Other differences between these two enzymes then became apparent, the most notable being in their substrate specificity[39,40,41]. Solutions of the enzyme having the lower pH optimum were found to act only on L-histidine. Solutions of the other enzyme were capable of decarboxylating a number of amino acids structurally related to histidine. These enzymes will be referred to as specific and non-specific histidine decarboxylase, respectively.

Non-specific Histidine Decarboxylase

Following the discovery of DOPA decarboxylase, which converts L-β-(3,4-dihydroxyphenyl)alanine to dopamine in mammalian tissues[42], a close similarity was noted in the distribution of histidine decarboxylase and DOPA decarboxylase activities. The possibility was considered that these two activities might reside in a single enzyme[43]. Later, an enzyme catalysing the decarboxylation of L-5-hydroxytryptophan (5-HTP decarboxylase) was detected in mammalian tissues[44] and it was found that the ratio of DOPA decarboxylase to 5-HTP decarboxylase activities in various organs of the rabbit and guinea-pig was roughly constant[45]. Thus it seemed possible that the histidine decarboxylase having its pH optimum in the range 8·0–9·5 might also be capable of decarboxylating DOPA and 5-HTP. Finally, when

an enzyme extract of guinea-pig kidney was purified fifty fold to a hundred fold, it was reported to decarboxylate not only these three amino acids but also many other natural and purely synthetic aromatic amino acids[46]. Thus it has been suggested that the histidine decarboxylase having its optimum activity in the pH range 8·0–9·5 should be designated aromatic L-amino acid decarboxylase*.

Apart from the similar distribution of the decarboxylase activities discussed above, further evidence that various aromatic L-amino acids are all decarboxylated by a single enzyme is based on : (*a*) failure to dissociate the activities during progressive purification of the enzyme; (*b*) the occurrence of competitive substrate inhibition; (*c*) the fact that all the decarboxylations are inhibited by the same inhibitors; (*d*) under conditions which lead to changes in the ability of a tissue to decarboxylate one substrate, parallel changes occur in the ability to decarboxylate the other substrates. In practice, most of this evidence has been obtained by the use of histidine, DOPA and 5-HTP as substrates.

By fractionating an extract of rabbit kidney cortex on a diethylaminoethyl-cellulose column, Rosengren[47] found that the chromatographic distribution of DOPA and 5-HTP decarboxylase activities was very similar. The ratio of the two activities throughout the peak was almost constant and equal to that observed in crude extracts. The peak also contained all the histidine decarboxylase activity of the extract. Similar results have been reported for the fractionation of guinea-pig kidney extracts[46]; Werle and Aures[48] have obtained from this tissue a purified preparation which decarboxylates DOPA and 5-HTP but not histidine. The conflicting observations of these two groups of workers may arise from differences in methodology, and the matter requires re-investigation.

The decarboxylation of DOPA by a rabbit kidney extract was found to be competitively inhibited by 5-HTP, and the decarboxylation of 5-HTP by the same extract was competitively inhibited by DOPA[47]. Kinetic studies of the mutual inhibitions[49] enabled the respective Michaelis and inhibitor constants, K_m and K_i, to be calculated, thus providing quantitative measurements of the affinities of these substances for the two enzymes (*Table 4.1*).

Table 4.1. Affinities of DOPA and 5-HTP for aromatic amino acid decarboxylase of rabbit kidney as shown by mutual inhibition studies at pH 7·5[47]

Amino acid	*Affinity* (mole/l)
DOPA as substrate of DOPA decarboxylase	$K_m = 7{\cdot}4 \times 10^{-5}$
DOPA as inhibitor of 5-HTP decarboxylase	$K_i = 7{\cdot}1 \times 10^{-5}$
5-HTP as substrate of 5-HTP decarboxylase	$K_m = 1{\cdot}8 \times 10^{-4}$
5·HTP as inhibitor of DOPA decarboxylase	$K_i = 1{\cdot}3 \times 10^{-4}$

* In accordance with the nomenclature recommended by the Enzyme Commission of the International Union of Biochemistry these enzymes have received the following systematic names:
DOPA decarboxylase; 3,4-dihydroxy-L-phenylalanine carboxy-lyase (EC4.1.1.26)
5-HTP decarboxylase; 5-hydroxy-L-tryptophan carboxy-lyase (EC4.1.1.28)
Histidine decarboxylase; L-histidine carboxy-lyase (EC4.1.1.22)
Aromatic L-amino acid decarboxylase; Aromatic L-amino acid carboxy-lyase.

The results show that if the extract contained two individual enzymes, DOPA decarboxylase and 5-HTP decarboxylase, then not only must DOPA have the same affinity for each enzyme, but 5-HTP must also have the same affinity for each enzyme. Alternatively the extract could contain only one decarboxylase capable of decarboxylating both DOPA and 5-HTP. As the second alternative is the more probable one, these results provide strong, though by themselves not conclusive, evidence that in the rabbit kidney extract one enzyme is responsible for the decarboxylation of both substrates.

In agreement with these findings, Rosengren also found that when *o*-tyrosine, *m*-tyrosine or caffeic acid were tested as inhibitors of the rabbit kidney preparation, the inhibition constants (K_i), and hence the affinities of each substance for the enzyme, were the same irrespective of whether DOPA or 5-HTP were used as substrate for the decarboxylase (*Table 4.2*).

Table 4.2. Comparative inhibition constants for the inhibition of DOPA and 5-HTP decarboxylases in a rabbit kidney extract at pH 7·5

Inhibitor	*Inhibitor constant* (K_i) (mole/l)	
	DOPA *as substrate*	5-HTP *as substrate*
o-Tyrosine	7×10^{-4}	7×10^{-4}
m-Tyrosine	4×10^{-4}	4×10^{-4}
Caffeic acid	3×10^{-4}	4×10^{-4}

(*From* Rosengren[47], *by courtesy of* Acta Physiologica Scandinavica)

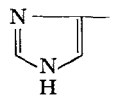
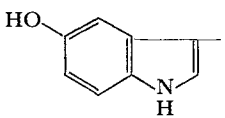
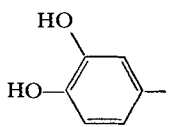
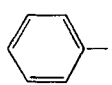
On the basis of similar mutual inhibition studies with DOPA and 5-HTP, Fellman[50] has concluded that both substrates are decarboxylated by the same enzyme in extracts of ox adrenal glands.

That not only DOPA decarboxylase and 5-HTP decarboxylase, but also the histidine decarboxylase (having its optimum activity in the range pH 8·0–9·5) are the same enzyme is also suggested by inhibition studies. α-Methyl-DOPA, for example, is a potent inhibitor of all three enzymes[51–53]; other α-methylamino-acids behave similarly[46].

Although various substances produce competitive inhibition of both the 5-HTP and DOPA decarboxylase activities of partially purified extracts of hog kidney[54,55] it has been stated that these compounds do not also inhibit histidine decarboxylase. This statement is misleading, however, as it refers to results obtained by earlier workers using a histidine decarboxylase of bacterial origin[56].

In rats treated with the liver carcinogen diethylnitrosamine (DENA), the mean activities of histidine decarboxylase (measured at pH 8·0), DOPA decarboxylase and 5-HTP decarboxylase in the liver were significantly lower than the corresponding mean values in the livers of control rats. In both the control and DENA-treated series, however, a positive correlation was found between the activities of these three enzymes[57]. When rats were maintained on a tryptophan-deficient diet, it was found[58] that the mean activities of histidine decarboxylase (pH 8·0), DOPA decarboxylase and 5-HTP decarboxylase in the livers of the tryptophan-deficient animals were significantly

Table 4.3. The substrate spectrum of non-specific histidine decarboxylase

Substrates R in $RCH_2 \cdot CH(NH_2) \cdot CO \cdot OH$		*Reactivity*	*Source of enzyme*	*Reference*
	Histidine	Low	See *Table 4.4*	See *Table 4.4*
	5-HTP	High	Rabbit kidney, guinea-pig kidney and other organs, ox adrenal, human phaeochromocytoma and argentaffinoma	45, 46, 50, 62, 68
	DOPA	Very high	Nerve tissue, rabbit kidney, guinea-pig kidney and other organs, ox adrenal, hog kidney cortex, human phaeochromocytoma and argentaffinoma	22, 45, 46, 50, 56, 62, 66, 68
	Phenylalanine	Moderate	Guinea-pig kidney	46

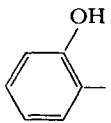
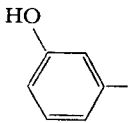
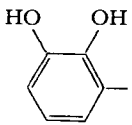
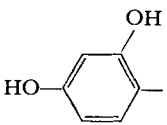
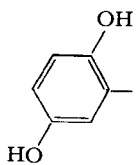
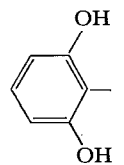
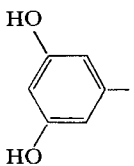
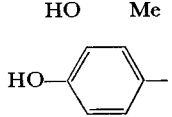
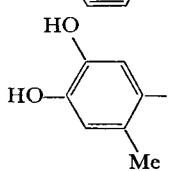
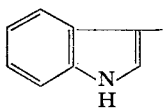
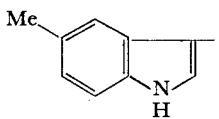
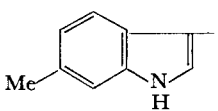
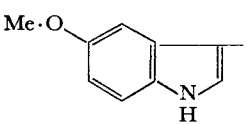
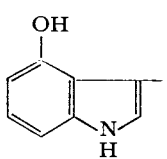
	*o*-Tyrosine	High	Guinea-pig kidney, ox adrenal, rat liver, hog kidney cortex, human phaeochromocytoma and argentaffinoma	46, 50, 56, 62, 67, 68, 69, 70
	*m*-Tyrosine	High	Guinea-pig kidney, ox adrenal, rat liver, hog kidney cortex, human phaeochromocytoma and argentaffinoma	46, 50, 56, 62, 67, 68, 70, 71
	2,3-DOPA	Very high	Hog kidney cortex, guinea-pig kidney	56, 70, 72, 73
	2,4-DOPA	Moderate	Hog kidney cortex, guinea-pig kidney	56, 70, 74
	2,5-DOPA	High	Guinea-pig kidney, rat liver, hog kidney cortex	56, 67, 70, 71, 74

Table 4.3. The substrate spectrum of non-specific histidine decarboxylase (*continued*)

Substrates R in $RCH_2 \cdot CH(NH_2) \cdot CO \cdot OH$	*Reactivity*	*Source of enzyme*	*Reference*
 2,6-DOPA	Moderate	Guinea-pig kidney, mouse brain	70, 73
 3,5-DOPA	Moderate	Guinea-pig kidney, hog kidney, mouse brain	70, 74
 2-Methyl-3,4-DOPA	Low	Hog kidney cortex	56
 6-Methyl-3,4-DOPA	Moderate	Hog kidney cortex	56

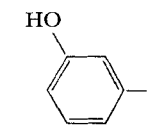
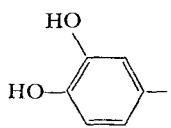
	β-(3,4,5-Trihydroxyphenyl)alanine	High	Guinea-pig kidney, mouse brain	70
	β-(3-hydroxy-4-methoxyphenyl)alanine	Very high	Guinea-pig kidney, mouse brain	70
	β-(3-hydroxy-4-methylphenyl) alanine	Very high	Guinea-pig kidney, mouse brain	70
	β-(2-hydroxy-3-methoxyphenyl)alanine	Low	Guinea-pig kidney, mouse brain	70
	β-(2-hydroxy-4-methoxyphenyl)alanine	Moderate	Guinea-pig kidney, mouse brain	70
	Kynurenine	Low	Guinea-pig kidney	46

Table 4.3. The substrate spectrum of non-specific histidine decarboxylase (*continued*)

Substrates R in $RCH_2 \cdot CH(NH_2) \cdot CO \cdot OH$	*Reactivity*	*Source of enzyme*	*Reference*
 Tryptophan	Moderate	Guinea-pig kidney	46
 5-Methyltryptophan	Very low	Guinea-pig kidney	46
 6-Methyltryptophan	Very low	Guinea-pig kidney	46
 5-Methoxytryptophan	Very low	Guinea-pig kidney	46
 4-Hydroxytryptophan	High	Guinea-pig liver, guinea-pig kidney, guinea-pig intestine, mouse brain	75

Substrates R in $RCH_2 \cdot CMe(NH_2) \cdot CO \cdot OH$			
	α-Methyl-DOPA	Low	Guinea-pig kidney	46, 52
	α-Methyl-*m*-tyrosine	Low	Guinea-pig kidney	46
	α-Methyltryptophan	Low	Guinea-pig kidney	46
	α-Methyl-5-HTP	Low	Guinea-pig kidney	46

Table 4.3. The substrate spectrum of non-specific histidine decarboxylase (*continued*)

Substrates R in R·CH(OH)·CH(NH$_2$)·CO·OH	*Reactivity*	*Source of enzyme*	*Reference*
 β-(3-Hydroxyphenyl)serine	Low	Nerve tissue, hog kidney cortex, ox, guinea-pig and rabbit kidney	56, 65, 66
 β-(3,4-Dihydroxyphenyl)serine	Low	Nerve tissue, hog kidney, ox and guinea-pig kidney, ox liver, guinea-pig intestine, ox adrenal medulla	54, 56, 65, 66, 74

lower than the corresponding activities in the livers of control rats. Nevertheless, a positive correlation was observed between the activities of the three enzymes in both the control and tryptophan-deficient series. It has also been shown that treatment of rats with thyroid hormones for several weeks leads to a decrease in the capacity of the liver to decarboxylate histidine[59], 5-HTP[59,60] and DOPA[61]. Since the three enzymic activities vary in parallel under these different physiological conditions, the presumption is strong that they are due to a single enzyme.

Thus there is much evidence to suggest that the histidine decarboxylase having its maximum activity in the pH range 8·0–9·5 is a single enzyme which can decarboxylate not only L-histidine, but also *L-β-(3,4-dihydroxyphenyl)*-alanine and L-5-hydroxytryptophan. Enzyme preparations which decarboxylate one or more of these three compounds have been found also to decarboxylate the substances listed in *Table 4.3*, thus providing support for the existence of a general aromatic amino acid decarboxylase. It is this enzyme which will be referred to as the non-specific histidine decarboxylase.

In some instances the results obtained by different groups of workers have been sufficiently at variance, particularly where weak substrates have been studied, for doubt to be cast on the existence of a general aromatic amino acid decarboxylase. Thus it has been claimed that some preparations which contain DOPA and 5-HTP decarboxylase activities do not decarboxylate histidine[10,62]. In these instances, the sensitivity or specificity of the analytical procedures are open to doubt, and the results require confirmation. In view of conflicting reports in the literature, further experiments should also be carried out to determine whether the mono- and dihydroxyphenylserines[56,63–66] are indeed substrates of non-specific histidine decarboxylase. The status of *p*-tyrosine also requires clarification; formerly it was not considered to be a substrate[50,56,67,68], but recent evidence suggests that it may, in fact, be decarboxylated[46,212].

Specific Histidine Decarboxylase

The histidine decarboxylase which has its maximum activity in the pH range 6·0–7·0 appears to be substrate-specific, acting only on L-histidine. This enzyme, which will be referred to as specific histidine decarboxylase, has been detected in various tissues, notably in mast cells, in the glandular portion of rat stomach, in rat foetal liver, and in certain tumours. Histidine decarboxylase activity which has been shown to be induced in tissues of various species when the animals are subjected to stressful stimuli[76] also has many of the properties of specific histidine decarboxylase. Some comparative properties of the two types of histidine decarboxylase derived from various mammalian sources are given in *Table 4.4*.

There is evidence that the specificity of the histidine decarboxylase of rat foetal liver may be even greater than was originally suspected. Thus, not only is its activity confined to L-histidine, but it appears to be further restricted to one particular ionic form of histidine. Over the pH range regarded as optimal for this enzyme, the substrate, histidine, exists as a mixture of ionic forms (*V, VI, VII, VIII*), and the concentration of each species present in a given solution can be calculated from the Henderson–Hasselbach equation. When the Michaelis constant for the decarboxylation was measured in terms of the

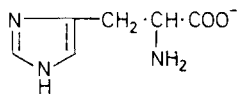
Table 4.4. Properties of histidine decarboxylases from various sources

Source of histidine decarboxylase	pH *for optimal activity*	*Affinity* for *histidine* (K_m) mole/l	*Substrates*	*Effect of benzene*	*Effect of α-Me-DOPA*	*Effect of α-Me-histidine*	*References*
Rat peritoneal mast cells	6·5–7·6			Strong inhibition			77
Mouse mastocytoma	6·0	5×10^{-4}	Histidine	None	No effect at $1{\cdot}0 \times 10^{-3}M$	No effect at $1{\cdot}0 \times 10^{-3}M$	40
Tumour (F-Hep) growing as S/C transplant in rat	6·5–7·0	$6{\cdot}8 \times 10^{-3}$	Histidine	Slight inhibition	No effect at $1{\cdot}0 \times 10^{-3}M$	50% inhibition at $1{\cdot}5 \times 10^{-3}M$	39, 78, 79, 80
Primary liver tumour produced in rat by DENA	6·0–6·5	$1{\cdot}2 \times 10^{-3}$	Histidine	None	50% inhibition at $2{\cdot}4 \times 10^{-2}M$	50% inhibition at $3{\cdot}6 \times 10^{-3}M$	57
Liver of foetal rat	6·4–7·2	$1{\cdot}0 \times 10^{-3}$	Histidine	None	No effect at $1{\cdot}0 \times 10^{-3}M$		16, 80, 81
Rat stomach (glandular portion)	6·8–7·2	$4{\cdot}5 \times 10^{-4}$	Histidine	Strong inhibition	No effect at $1{\cdot}0 \times 10^{-3}M$	50% inhibition at $7{\cdot}5 \times 10^{-3}M$	20, 82, 83 255

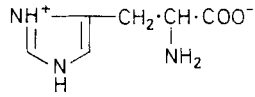
Inducible enzyme of G.P. lung or mouse liver	7·4–8·0		Histidine	Weak inhibition	<40% inhibition at $10^{-4}M$	>90% inhibition at $10^{-4}M$	76, 84
Bone marrow of rat	6·3–6·8		Histidine				22
Guinea-pig kidney	9·0–9·5	$5{\cdot}0 \times 10^{-2}$	Histidine DOPA 5-HTP, etc.	Stimulation	100% inhibition at $1{\cdot}0 \times 10^{-3}M$	No effect at $1{\cdot}0 \times 10^{-3}M$	2, 39, 40, 79
Rabbit kidney	8·0	$1{\cdot}0 \times 10^{-1}$	Histidine DOPA 5-HTP, etc.	Stimulation			20, 41, 47
Liver of normal rat	8·0	$5{\cdot}3 \times 10^{-1}$	Histidine DOPA 5-HTP	Stimulation	50% inhibition at $9{\cdot}0 \times 10^{-6}M$	50% inhibition at $1{\cdot}1 \times 10^{-2}M$	57, 80, 82
Mouse kidney	7·0		Histidine	None	Weak inhibition	Strong inhibition	85, 86, 87, 88
Foetal mouse skin	7·0		Histadine	None	Weak inhibition	Strong inhibition	85, 86, 87, 88

total histidine concentration, it was found to vary markedly as the pH was altered[16]. Similarly, the Michaelis constant measured in terms of the concentration of the ionic species *VI*, *VII* and *VIII* varied as the pH was altered. However, when based on the concentration of anionic histidine, the Michaelis constant showed little variation.

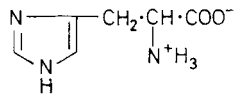
These observations suggest that the true substrate for the specific histidine decarboxylase of rat foetal liver is the anionic form of histidine (*V*). It should be noted that this interpretation involves the assumption that the enzyme



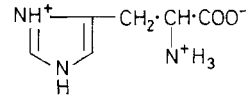
(V)



(VI)



(VII)



(VIII)

itself remains unaffected by pH changes. Since alterations in pH undoubtedly affect the enzyme[16], the above conclusion may ultimately require some modification.

It has recently been reported that extracts of foetal rat liver can decarboxylate not only histidine but also DOPA, 5-HTP and other aromatic L-amino acids[212]. This may mean that the specific histidine decarboxylase has a wider substrate specificity than was originally believed, or that the extract contains a mixture of the specific and non-specific enzymes. The rat glandular stomach provides an example of a tissue in which the presence of both enzymes has now been demonstrated[215].

THE DISTRIBUTION OF HISTIDINE DECARBOXYLASES

As already mentioned, histidine decarboxylase is present in certain bacteria[6]. Although Werle and co-workers[89,90] found histamine itself in a number of higher plants, they were unable to detect histidine decarboxylase in the plant extracts. They were, however, able to demonstrate the formation of histamine by intact spinach seedlings growing in a nutrient solution containing histidine. The high histidine decarboxylase activity of spinach seedlings was later confirmed, but negligible activity was observed in seedlings of ten other plant species examined at the same time[216]. Histidine decarboxylase activity has also been found in certain leguminous plants[91], and in cotton seedlings, *Gossypium hirsutum*[92]. While the specificity of plant histidine decarboxylase has not been systematically studied, the limited evidence available suggests that it is not identical with plant DOPA decarboxylase.

Histidine decarboxylase occurs in many animal tissues. High activity has

been found in such comparatively simple organisms as the sea-anemone, *Aiptasia tagetes*, and in the parasitic worm, *Mesocoelium monodi*[93,217]. As the enzyme extracted from these is active over an unusually wide pH range (4–9), and as it is not activated by the addition of benzene or pyridoxal phosphate, it may belong to a type different from those hitherto described.

Little information is available with regard to the occurrence of histidine decarboxylase in insects. It has, however, been detected in the venom, sting and gut of the bee[94]. The enzyme from the sting organ was examined in some detail, and was found to have its optimum activity at pH 8. Although it was stated to be stereospecific, i.e. acting on L-histidine but not on D-histidine, its action on other aromatic L-amino-acids was not investigated. The high pH optimum is similar to that of the non-specific histidine decarboxylase of mammalian origin, and further study of the enzyme from bees, with particular reference to its substrate and inhibitor specificities, would therefore be of considerable interest.

Although histidine decarboxylase has been detected in many mammals, its distribution in the tissues varies markedly from species to species. Sex differences in the distribution of histidine decarboxylases are uncommon, the most striking example being found in the mouse where the activity in the kidney of the female greatly exceeds that in the male. The properties of the mouse kidney enzyme indicate that it is a specific histidine decarboxylase[85–88]. Owing to the various methods and conditions employed for the measurement of histidine decarboxylase activity, the levels of the enzyme reported for a given tissue by different workers may vary considerably. The discrepancies, which are greatest for tissues in which the decarboxylase content is low, may be attributable to failure to ensure that the L-histidine used as substrate is histamine-free, variations in the amount of the co-enzyme pyridoxal phosphate added, use of different substrate concentrations, and differences in the pH of the incubation medium. When various groups of workers use different conditions of pH in measuring histidine decarboxylase activity, it also becomes difficult to decide whether the observed activity is that of the specific or the non-specific enzyme, particularly when low substrate concentrations are employed.

Lack of agreement between published results is sometimes due to the fact that certain workers add benzene to the reaction medium when measuring histidine decarboxylase activity. Benzene is known to have a stimulating effect on non-specific histidine decarboxylase[13,95] and, when added to incubation mixtures, has led to the detection of activity in tissues where its presence had not previously been reported[82]. This practice of adding benzene to the incubation mixtures has been criticized as unphysiological[96]; nevertheless, all *in vitro* experiments are unphysiological to some extent. The histidine decarboxylase activities observed in the presence of benzene differ from organ to organ and from species to species. These differences require explanation, and, until the underlying mechanism has been clarified, it would be unwise to assume that they have no physiological significance.

The main sites of occurrence of histidine decaboxylases in normal adult mammalian tissues are shown in *Table 4.5* (rodents) and *Table 4.6* (man and domestic animals). All references therein relate to tissues in extracts of which the actual decarboxylation of histidine has been investigated. References to

Table 4.5. Principal locations of histidine decarboxylases in mammalian tissues (rodents)

Organ	*Rat*		*Mouse*		*Guinea-pig*		*Rabbit*		*Hamster*		*Ferret*	
	Activity	*Ref.*	*Activity*	*Ref.*	*Activity*	*Ref.*	*Activity*	*Ref.*	*Activity*	*Ref.*	*Activity*	*Ref.*
Liver	1N	13, 57, 82, 98	1N	13, 98, 99	2N	13, 98, 99, 218, 253	2N	13, 98, 99	1	98, 99	0	98
Kidney	1N	82	1S*	13, 85, 86, 87, 88, 98, 99	3N	13, 98, 99, 218, 253	3N	13, 98, 99	2	98, 99, 102	0	98
Lung	0	13, 82	0	13	1N	13, 218	0	13, 99				
Stomach¶	2S†	13, 82, 83, 98	1‡	13, 87, 98, 100	1N	13, 40, 98, 215, 218, 219 253	1	13, 98, 101, 215	1	98, 102	0	98
Small intestine	1N	13, 82	1N	13	2N	13, 218, 253	0	13				
Sub-maxillary gland			1N	101								

Histidine decarboxylase (HD) activity is expressed in arbitrary units: 1 = low activity; 2 = moderate activity; 3 = high activity. The probable nature of the enzyme is indicated by N = non-specific HD, S = specific HD; where no letter is given the nature of the enzyme is in doubt.

* Is higher in female than in male and increases to 3S in pregnant female[87,88].

† Is higher in pregnant female[176] and shows increase in fed animals[177].

‡ Is higher in pregnant female and shows a hundred fold increase in fed as compared with starved animals[100].

¶ Activity varies from one part of the stomach to another, and it is confined mainly to the mucosa. Activity in the rat stomach is confined mainly to the glandular mucosa.

Table 4.6. Principal locations of histidine decarboxylases in mammalian tissues (man and domestic animals)

Organ	*Man*		*Dog*		*Cat*		*Ox*		*Pig*	
	Activity	*Ref.*	*Activity*	*Ref.*	*Activity*	*Ref.*	*Activity*	*Ref.*	*Activity*	*Ref.*
Liver	1N*	103	0	13, 98	0	13, 98				
Kidney	1N*	103	0	13, 98, 99	0	13, 98, 99	0	99		
Lung	0*	103	0	13	0	13				
Stomach†	1	219	1	219	0	13, 98			1	219
Small intestine			0	13	0	13				
Brain	0*	103	1	46, 104	1	104	1N	105	1	104
Spinal Cord							1N	66, 105		
Splenic nerve							1N	66		
Stellate ganglion							1N	66		
Submaxillary gland			1N	101			1N	101	1N	101

N = non-specific HD; where no letter is given the nature of the enzyme is in doubt. Activity expressed in arbitrary units.
* New Born.
† Activity in the stomach of these species is very low and is normally detectable only in extracts of the mucosa.

observations made by isotopic methods are included only where the degree of decarboxylation is sufficient to be detectable by non-isotopic procedures also. No account has been taken of tissues in which the presence of aromatic L-amino-acid decarboxylase has been inferred from their DOPA or 5-HTP decarboxylase activity. Examination of *Tables 4.5* and *4.6* reveals that most rodents possess several potent sites of histidine decarboxylation, notably in liver, kidney and stomach, whereas in most domestic animals the enzyme is either absent or is present only in small amounts. The possible significance of this observation is discussed later in relation to the physiological function of histidine decarboxylase. Histidine decarboxylase activity is also found in some normal (including foetal) and pathological tissues which are characterized by a rapid rate of growth. The enzyme from these sources will be considered later in relation to the possible physiological functions of histidine decarboxylases.

The relationship of the histamine content of a tissue to its histidine decarboxylase activity has been the subject of several investigations[13,97]. Apart from the difficulty already mentioned of correlating the results of histidine decarboxylase measurements made by different groups of workers,

Table 4.7. Relationship between histidine decarboxylase activity of tissues and their histamine content[13,82,101,108,109]

Tissues containing			
High HD *High* H	*Low* HD *Low* H	*High* HD *Low* H	*Low* HD *High* H
Rat stomach (Glandular)	Rat kidney	Rabbit kidney	Rat abdominal skin
	Adult rat liver	Rabbit liver	
	Rat lung		Rat duodenum
Rabbit stomach (Fundus)	Rabbit small intestine	Guinea-pig kidney	Rat ileum
		Guinea-pig liver	
	Rabbit lung		Rat jejunum
	Rabbit stomach (Pylorus)		Rat stomach (non-glandular)
			Guinea-pig lung

H = histamine; HD = histidine decarboxylase

a similar difficulty arises in interpreting the results of tissue histamine determinations. Extracts prepared with saline or Tyrode solution contain histamine which is not bound to tissue components, while those made with trichloroacetic acid or perchloric acid contain the total extractable histamine including the bound histamine. Histamine contents measured by different extraction procedures may therefore show discrepancies especially where a considerable proportion of the histamine is present in a bound form.

It is possible for a tissue to have a low or impaired histamine-forming capacity (HFC) and yet to have a high histamine content by virtue of its ability to store the amine. Thus in rats subjected to prolonged inhibition of histamine formation by means of a pyridoxal-deficient diet[8], the HFC in abdominal skin, tongue and lung was reduced to less than 10 per cent of normal without diminishing the histamine content. In the gastric mucosa,

on the other hand, a similar reduction in the HFC produced a substantial decrease in the histamine content. There is, therefore, no general relationship between the histidine decarboxylase activity of a tissue and the histamine content. This is shown in *Table 4.7* which has been compiled from published figures obtained by different workers using similar conditions for histidine decarboxylase measurements and extraction procedures suitable for the determination of total tissue histamine.

THE MECHANISM OF ACTION OF HISTIDINE DECARBOXYLASES

Co-enzyme Requirement

Werle[106] suggested that the active centre of histidine decarboxylase might contain a carbonyl group, since the enzyme was inhibited by reagents such

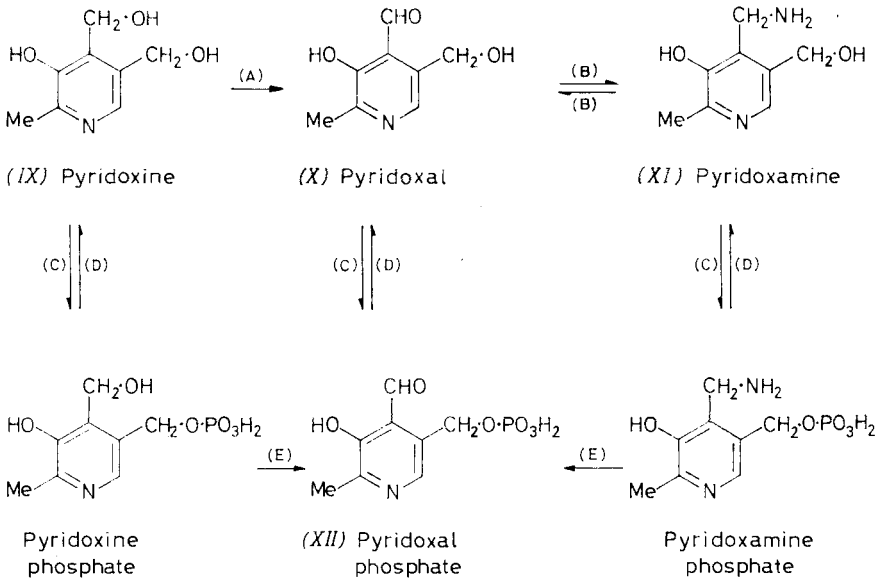


Figure 4.1. Possible pathways for the formation of pyridoxal phosphate *in vivo* (adapted from Wada and Snell[210])

(A) Pyridoxine oxidase
(B) Pyridoxamine transaminase
(C) Pyridoxal kinase
(D) Phosphatase
(E) Pyridoxine phosphate oxidase

as cyanide, bisulphite, hydroxylamine, semicarbazide and phenylhydrazine. Subsequently, pyridoxal phosphate (*XII*) (*Figure 4.1*) was shown to be the co-enzyme of several bacterial amino acid decarboxylases[107,244]. If pyridoxal phosphate was also the co-enzyme for mammalian histidine decarboxylases, this would account for the presence of the carbonyl group postulated by Werle.

Although pyridoxal phosphate is now commonly accepted to be the co-enzyme of the mammalian histidine decarboxylases, the available evidence

is not conclusive, especially in the case of the non-specific histidine decarboxylase. In order to demonstrate that pyridoxal phosphate is acting as a co-enzyme, it is necessary to remove it from the holo-enzyme and to show that the resultant apo-enzyme is devoid of activity. Subsequent addition of the co-enzyme should then restore the activity. Such a proof is not always possible, especially if the apo-enzyme has a very high affinity for the co-enzyme as in the case of the non-specific histidine decarboxylase of guinea-pig kidney[220]. However, pyridoxal phosphate is less strongly bound by the specific enzyme. Thus Burkhalter[81] removed the co-enzyme from the specific histidine decarboxylase of foetal rat liver by gel filtration. The resultant extract was inactive and activity was restored by the addition of pyridoxal phosphate.

When purified, the DOPA decarboxylase of rat liver has an absorption spectrum similar to that of other pyridoxal-dependent enzymes. In this case, the co-enzyme seems to be very tightly bound to the apo-enzyme, but addition of an excess of pyridoxal phosphate still causes an increase in the enzyme activity[110]. It was therefore suggested that pyridoxal phosphate is a prosthetic group of this enzyme, and that when present in excess it acts as a co-enzyme. The 5-HTP decarboxylase of rat kidney was found to be potentiated by pyridoxal phosphate, but the effect was shown only when the tissue had been repeatedly frozen and thawed[111]. These observations provide some evidence that pyridoxal phosphate is the co-enzyme for non-specific histidine decarboxylase.

Pyridoxine (Vitamin B_6) (*IX*), pyridoxal (*X*), and pyridoxamine (*XI*), which are normally available in the diet, must be converted *in vivo* to the active co-enzyme pyridoxal phosphate (*XII*) as shown in *Figure 4.1*. Inhibition of pyridoxal phosphate production *in vivo* results in reduced activity of pyridoxal-dependent enzymes. The extent of the inhibition may vary from one enzyme system to another, depending on the affinities of the apo-enzymes for the co-enzyme.

Non-enzymic Decarboxylation of Amino Acids

Pyridoxal phosphate is a co-enzyme for numerous enzymes, notably amino acid decarboxylases, amino acid transaminases, histaminase and probably diamine oxidase[112,113,114]. As most of the evidence on which the mechanism of action of pyridoxal-dependent enzymes is based has been obtained from studies of the non-enzymic interaction of pyridoxal with amino acids, these non-enzymic reactions will be considered first in some detail.

Snell and Rannefeld[115] discovered that when pyridoxal was autoclaved with amino acids, the resultant material had growth-promoting properties similar to those of pyridoxamine. This suggested that pyridoxal might have been converted to pyridoxamine, and this was later confirmed[116,117]. The pyridoxal had catalysed the de-amination of the amino acids. It was subsequently shown that pyridoxal catalyses many reactions involving amino acids, including de-amination, racemization, decarboxylation, $\alpha\beta$-elimination and cleavage. Addition of appropriate multivalent metal ions increased the rate of de-amination and $\alpha\beta$-elimination reactions, but inhibited decarboxylation.

General mechanisms (*Figure 4.2*) were then proposed to explain the above

results[118,119]. These mechanisms suggested for non-enzymic reactions are likely to apply also, with minor modifications, to the corresponding reactions catalysed by pyridoxal-dependent enzymes. The first stage of the reaction is considered to be the formation of a Schiff base (*XIII*) from the amino acid and pyridoxal. The production of (*XIII*) is characterized by the rapid appearance of a yellow colour. Schiff bases of pyridoxal have been isolated[120], and equilibrium constants for their formation have been determined[121]. Pyridoxal phosphate also forms Schiff bases, and detailed kinetic studies of the reaction have been made by spectroscopic methods[122,123].

The next step in the reaction scheme (*Figure 4.2*) is considered to be the

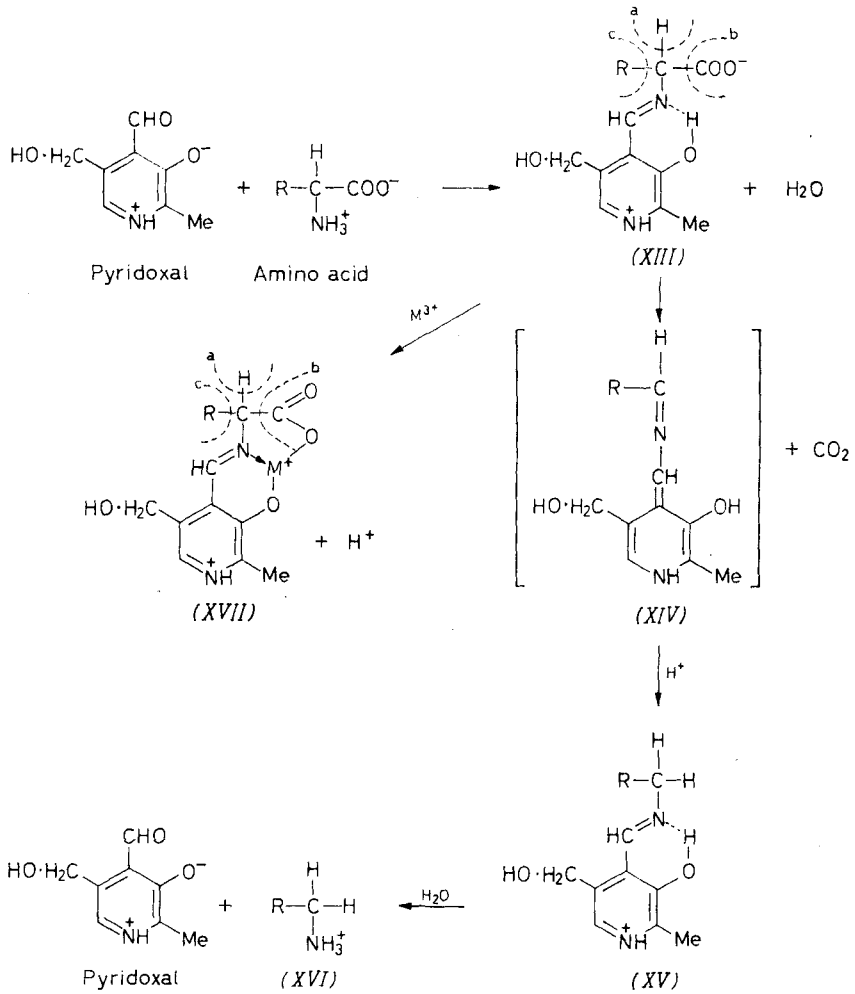
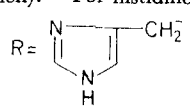


Figure 4.2. Mechanism for the non-enzymic, pyridoxal-catalysed decarboxylation of amino acids (after Snell).[118] For histidine,



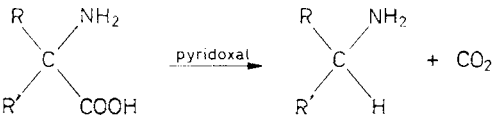
weakening of one of the bonds (*a*), (*b*) or (*c*). The system of conjugated double bonds present in the structure (*XIII*), enables the electrophilic nitrogen atom of the pyridine ring to withdraw electrons from the nitrogen atom which was formerly the α-nitrogen of the amino acid. The bonds (*a*), (*b*) and (*c*) are thus weakened; cleavage of these bonds then results respectively in the release of a proton, or of carbon dioxide, or of a carbonium ion. Subsequent rearrangements and hydrolysis would lead to racemization, de-amination, αβ-elimination, cleavage or decarboxylation of the amino acid. In the case of decarboxylation, the Schiff base (*XIII*) loses carbon dioxide to form the intermediate structure (*XIV*). Addition of a proton leads to structure (*XV*) which is the Schiff base of the amine produced by decarboxylation of the amino acid. Hydrolysis of this Schiff base gives the free amine (*XVI*), with regeneration of pyridoxal. In the particular case where R = 4(5)-imidazolylmethyl, *Figure 4.2* represents the non-enzymic decarboxylation of histidine. If metal ions are added to an aqueous solution containing pyridoxal and an amino acid, a complex (*XVII*) may be formed; there is spectroscopic evidence for the formation of such metal complexes of Schiff bases[124]. The metal ion may stabilize the Schiff base[118] and, by maintaining a planar structure, facilitate the electron transfers which are required for the subsequent reactions. The metal ion would also be expected to reinforce the effect of the electrophilic nitrogen of the pyridine ring. However, the metal ion is not necessary for detectable catalytic activity, the electrophilic group in the ring being sufficient.

As already mentioned, certain multivalent metal ions inhibit the non-enzymic, pyridoxal-catalysed decarboxylation of amino acids. It has been suggested[125] that this inhibition may occur, as the spatial orientation of the carbonyl group and the bonding of this group to the metal ion lead to a reduced tendency for carbon dioxide to escape from the chelation complex (*Figure 4.2*; *XVII*). At the same time, the electrophilic character of the carbonyl group is enhanced by chelate formation, thus producing increased labilization of bonds (*a*) and (*c*), and increasing the rate of the other possible reactions.

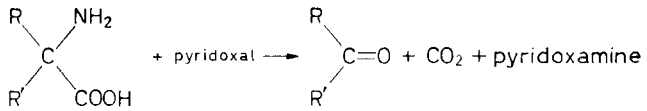
Studies of the non-enzymic catalytic effects of various compounds related to pyridoxal show that certain structural features are necessary for catalytic activity[118]. The formyl group is essential; it should be situated *ortho* or *para* to a strongly electronegative centre and *ortho* to a free phenolic hydroxyl group. The 5-hydroxymethyl group of pyridoxal is not required for non-enzymic catalysis; in fact, the 5-hydroxymethyl group reduces the catalytic activity of pyridoxal by forming a hemi-acetal with the formyl group[126]. Phosphorylation of the 5-hydroxymethyl group, or its replacement by a methyl group, prevents hemi-acetal formation and thus favours non-enzymic catalysis. The 2-methyl group of pyridoxal is not required for activity.

A detailed theoretical analysis of non-enzymic pyridoxal-catalysed reactions has been carried out by the molecular orbital method[127]. An interesting result of this analysis was the emphasis placed on the increased resonance energy of structures such as (*XIV*) compared with that of the original Schiff base (*XIII*). It was suggested that this increase in resonance energy is the main reason for the labilization of the bonds (*a*), (*b*) or (*c*), after Schiff base formation.

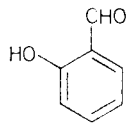
The above general mechanism for non-enzymic, pyridoxal-catalysed processes was derived mainly from a study of transamination reactions. Nevertheless, non-enzymic, pyridoxal-catalysed decarboxylations have been reported, for example, that of histidine to histamine[2]. The following pyridoxal-catalysed, non-enzymic decarboxylations of α-aminoisobutyric acid ($R = R^1 = CH_3$), α-methylserine ($R = CH_2OH$; $R^1 = CH_3$) and α-phenylgylcine ($R = H$; $R^1 = C_6H_5$) have also been described[125]:



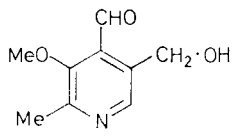
and



The first reaction is equivalent to enzymic decarboxylation; however, no enzymic equivalent of the second reaction is known. Both were inhibited by the presence of those metal ions which catalyse the other possible pathways covered by the general scheme (*Figure 4.2*). When salicylaldehyde (*XVIII*) or 4-formyl-5-hydroxymethyl-3-methoxy-2-methylpyridine (*XIX*) was used



(XVIII)



(XIX)

in place of pyridoxal, no decarboxylation was observed. It was concluded that the structural requirements for non-enzymic catalysis of amino acid decarboxylation resemble those for transamination.

Enzymic Decarboxylation

The probable mechanism of the enzymic decarboxylation of histidine can, at present, only be inferred from studies of the non-enzymic reactions discussed in the previous section, and from what is known of the mechanism of action of other pyridoxal phosphate-dependent enzymes.

A mechanism for enzymic decarboxylation of amino acids was suggested by Werle and Koch[2] and is shown in *Figure 4.3*. According to this mechanism, the α-hydrogen atom of the amino acid is lost in the reaction (*XX*)→(*XXI*) prior to decarboxylation. On the other hand, in Snell's mechanism, which has already been considered (*Figure 4.2*), the α-hydrogen atom remains bonded to the carbon atom throughout the series of reactions (*XIII*)→(*XIV*)→(*XV*)→(*XVI*). In order to distinguish between these mechanisms, the bacterial decarboxylation of the amino acids lysine, tyrosine and glutamic

acid was carried out in deuterium oxide[128]. It was found that the resultant amines contained only one atom of deuterium, bonded to the α-carbon, per molecule of base. This observation can be explained by the general mechanism (*Figure 4.2*), but not by the alternative one (*Figure 4.3*), and it indicates that decarboxylation can occur even when there is no hydrogen atom directly attached to the α-carbon atom of the amino acid. In agreement with this view, it has been found that pyridoxal can catalyse the non-enzymic decarboxylation of amino acids containing α-alkyl substituents to yield the corresponding α-substituted amines[125], and that α-methyl amino acids, for example α-methyl-DOPA, can undergo enzymic decarboxylation[46].

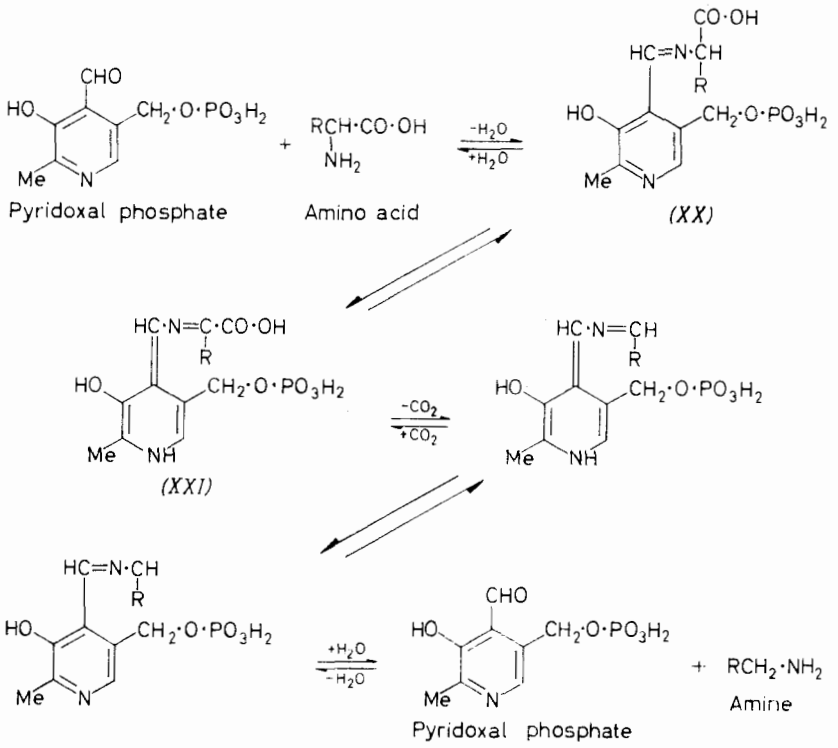
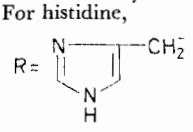


Figure 4.3. Mechanism for the enzymic decarboxylation of amino acids proposed by Werle and Koch[2]. For histidine,



It is also of interest that the decarboxylation of lysine and tyrosine by bacterial enzymes has been shown to be reversible[129,130]. In a reaction mixture consisting of enzyme, $^{14}CO_2$ and amino acid or amine the ^{14}C was incorporated into the carbonyl group of the amino acid. The position of equilibrium for the production of the amine from the amino acid was found to lie well over towards the amine. With lysine, for example, the equilibrium constant (amine) · (HCO_3^-)/(amino acid) was in the range 500 to 1000.

The nature of the interaction between the substrate and the apo-enzyme must control the substrate specificity and the optical specificity of the enzymic reactions, and must also determine which of the bonds *a*, *b* or *c* (*Figure 4.2*) is broken in any given case. A minimum of three points of attachment of the substrate to the active centre would account for the optical specificity.

An understanding of the mode of binding of the co-enzyme to the apo-enzyme is also required. The ability of various compounds, structurally related to pyridoxal phosphate, either to combine with the apo-enzyme to form an active holo-enzyme or to inhibit the formation of an active holo-enzyme, can be used to assess the affinities of these substances for the apo-enzyme. Such studies indicate that the phosphorylated 5-hydroxymethyl group of pyridoxal plays a major role in the binding of pyridoxal phosphate to the apo-enzyme, and that the free phenolic group also contributes to the binding[107].

It has been claimed that one of the pyridoxal phosphate-dependent enzymes, glutamic-oxaloacetic transaminase, is not inactivated when it is incubated with excess cyanide and then dialysed[131]. Under these conditions pyridoxal phosphate is inactivated by conversion to the cyanhydrin, but this cyanhydrin did not prevent reactivation of the apo-enzyme when free pyridoxal phosphate was subsequently added. This suggests that the cyanhydrin does not combine with the apo-enzyme, and that pyridoxal phosphate itself is attached to the apo-enzyme by the aldehyde group. Stronger evidence of the importance of the aldehyde group in binding the co-enzyme to the apo-enzyme is provided by the absorption spectra of several enzymes, including glutamic-aspartic transaminase and glutamic acid decarboxylase, which, like Schiff bases of pyridoxal phosphate, show an absorption band at 420–430mμ[132]. There is evidence that in pyridoxal-dependent enzymes the cofactor is attached to the apo-enzyme by forming an imino linkage with the ε-amino group of a lysine unit in the enzyme molecule[221,222].

It might seem that the binding of the aldehyde group to the apo-enzyme would render the non-enzymic mechanism (*Figure 4.2*) inapplicable to enzymic reactions. This is not so, however, since Schiff bases of pyridoxal phosphate are highly reactive, and they can, under certain conditions, react more rapidly with an amino acid than does the free aldehyde[133,134,135]. The co-enzyme may, therefore, be bound to the apo-enzyme by the phosphate, aldehyde and phenolic groups as shown in *Figure 4.4a*. The imino linkage (Schiff base) formed between the aldehyde group and the apo-enzyme may then react with the substrate as indicated in *Figure 4.4b*. Decarboxylation probably then proceeds by the mechanism suggested by Westheimer[136], the active centre reverting to the structure shown in *Figure 4.4a* once the decarboxylation has taken place.

INHIBITION OF HISTIDINE DECARBOXYLATION *in vitro*

Methods of Producing Inhibition

The decarboxylation of histidine at any particular site depends on the presence of appropriate amounts of substrate, co-enzyme and apo-enzyme. Any interference with the supply or functioning of these components could lead to inhibition of histamine production.

A study of the decarboxylation of histidine by solutions of the enzyme is simple enough in principle, the supply of co-enzyme, substrate and apo-enzyme being under the control of the experimenter. The action of the enzyme on the substrate may be inhibited by displacing the co-enzyme with a related compound which either cannot function, or functions less efficiently,

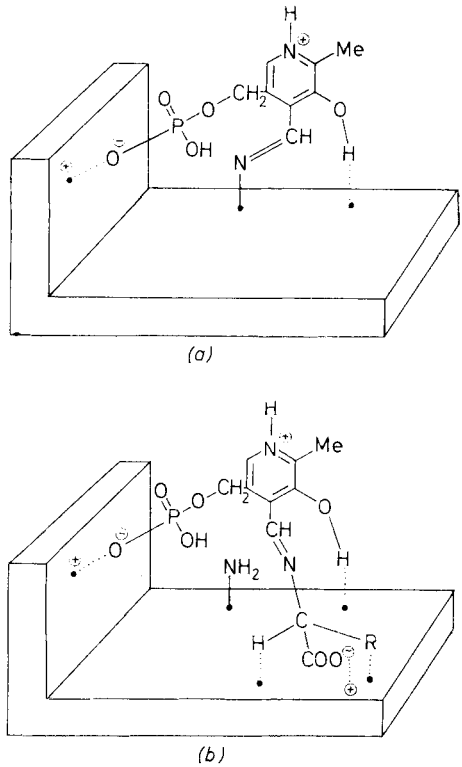
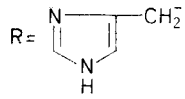


Figure 4.4. (*a*) Diagrammatic representation of the co-enzyme pyridoxal phosphate attached to an apo-enzyme by the phosphate, phenol and aldehyde groups. The aldehyde group has formed a Schiff base by interaction with an amino group of the apo-enzyme molecule; (*b*) Interaction between apo-enzyme, co-enzyme and an L-amino acid substrate. The amino group of the substrate has attacked the Schiff base linkage between the pyridoxal phosphate and the apo-enzyme, and has itself in turn formed a Schiff base with the aldehyde group of the pyridoxal. For histidine decarboxylase,



as a co-enzyme. Alternatively, some agent can be used which inactivates the co-enzyme by chemical reaction. Similar considerations apply to the substrate. It may be displaced from the active centre of the enzyme by a structurally related amino acid. If the analogue is decarboxylated by the enzyme, then it will behave as a competitive substrate, but the product, of course, will not be histamine. If the analogue is not decarboxylated by the enzyme, then it acts as a competitive inhibitor. Certain less specific methods of

inhibition, such as the use of proteolytic enzymes, thiolreactive reagents, and metal-chelating agents, may be used to provide information regarding the active centre of the enzyme.

Many of the results of inhibiton studies *in vitro* obtained by different workers, are not directly comparable because of differences in the incubation conditions employed. Measurements of the percentage inhibition produced by an arbitrary concentration of a particular inhibitor are of only limited value. Ideally, the results of inhibition studies should be expressed as affinity constants of the inhibitors for the enzyme. An alternative is to quote the concentrations of the inhibitors which are required to produce 50 per cent inhibition.

The use of tissue slices for experiments on histidine decarboxylation introduces the additional problem of the access of substrate, co-enzyme and inhibitors into the cells. In this connection, it should be noted that in practice the specificity of an enzyme within a cell may be increased by the specificity of the substrate-transporting system. Similar considerations apply to the *in vivo* inhibition of histidine decarboxylases; there is, however, the additional possibility of modifying production of the apo-enzyme either by restricting the supply of amino acids or by altering the hormonal state of the animal.

Inhibition of Non-specific Histidine Decarboxylase

Carbonyl reagents, including cyanide, hydroxylamine, semicarbazide, hydrazine and substituted hydrazines inhibit non-specific histidine decarboxylase by combining with the co-enzyme pyridoxal phosphate. Such compounds, of course, inhibit other pyridoxal-dependent enzymes. A list of these and other compounds which inhibit non-specific histidine decarboxylase has been compiled by Schayer[245].

Compounds related either to histidine or to DOPA have been used to assess the relative extent to which the ring system and the side chain contribute to the affinity of inhibitors for the histidine decarboxylase of guinea-pig kidney[2,137]. The relative inhibitory potencies of the compounds tested was in the order DOPA>catechol>pyrogallol>hydroquinone>resorcinol>tryptophan>indole>tyrosine>phenol>imidazole>alanine. It was concluded that two phenolic hydroxyl groups situated *ortho* to one another may play a major part in binding the inhibitor to the apo-enzyme. In agreement with this conclusion, the inhibitory potency of α-methyl-DOPA[51] and of *ortho*-dihydroxy-substituted flavones[138] is greatly diminished by methylation of these hydroxyl groups. It is possible that the flavones may be converted to quinones which could then be the inhibitors. Such quinones would be likely to react with any thiol and amino groups present in the enzyme molecule.

Several substituted histidines (*Table 4.8*) have been tested as inhibitors of the histidine decarboxylase of guinea-pig kidney[53]. From a consideration of the potencies of the substances tested, it was suggested that increasing the acidity of the nitrogens of the imidazole ring tended to produce stronger inhibitors. Similar studies on the inhibition of histamine formation by compounds related to DOPA or 5-HTP (*Table 4.8*) showed that α-methyl-DOPA and DOPA are good inhibitors[53,79]; only the L-form of α-methyl-DOPA is an effective inhibitor of L-amino acid decarboxylase[139]. The

O-substituted hydroxylamines (*XXII*, *XXIII*, and *XXIV*) and the substituted hydrazine (*XXV*) were the most potent inhibitors of non-specific histidine decarboxylases revealed by these studies (*Table 4.8*).

We have seen that the non-specific histidine decarboxylase may be identical

Table 4.8. Inhibition of histidine decarboxylases[53]

The inhibitor potencies of the various compounds are given as C_{50} values. C_{50} is the molar concentration of the inhibitor which is required to reduce the initial velocity of the uninhibited reaction by 50 per cent. All experiments were at 36°C. The guinea-pig kidney enzyme (GPK) was studied at pH 8·0 with L-histidine (32×10^{-3}M) as substrate. The rat hepatoma enzyme (F-Hep) was studied at pH 6·8 with L-histidine ($0{\cdot}64 \times 10^{-3}$M) as substrate.

Compound	GPK	F-Hep
	$C_{50} \times 10^4$	$C_{50} \times 10^4$
DL-α-Methyl-DOPA	0·01	100
L-DOPA	0·2	9
DL-DOPA	0·2	4·5
L-2,4-Di-iodohistidine	40	—
D-2,4-Di-iodohistidine	40	—
L-Monoiodohohistidine	50	—
DL-2-Methylhistidine	60	20
D-Histidine	600	625
DL-4-Methylhistidine	380	—
DL-β-(1-Methylimidazol-4-yl)alanine	420	—
L-β-(1-Methylimidazol-5-yl)alanine	910	—
Catechol	1·8	65
Imidazole	800	1600
4-Methyl-5-nitroimidazole	34	160
Imidazole-4(5)-carboxylic acid	30	150
4(5)-Nitroimidazole-5(4)-carboxylic acid	65	300
Imidazole-4(5)-carboxyhydrazide	20	15
4(5)-Nitroimidazole-5(4)-carboxyhydrazide	6·5	0·75
L-Histidine hydrazide	0·85	2
Hydrazine hydrate	0·35	0·1
DL-5-Hydroxytryptophan	0·65	75
DL-α-Methyl-5-hydroxytryptophan	0·075	1
DL-α-Methylhistidine	150	15
Salicylic acid	35	30
NSD 1024 (*XXII*)	0·0052	0·006
NSD 1055 (*XXIII*)	0·0027	0·0014
Imidazol-4(5)-methylhydroxylamine (*XXIV*)	0·065	0·0021
NSD 1034 (*XXV*)	0·0025	0·0098

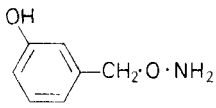
with the enzyme which decarboxylates DOPA and 5-HTP. It would not be surprising, therefore, if substances which inhibit the decarboxylation of DOPA or 5-HTP proved also to be effective inhibitors of histidine decarboxylase; data compiled by Clark[140] suggest that this is so.

Inhibition of Specific Histidine Decarboxylase

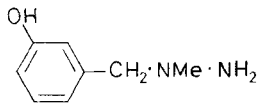
The effect of inhibitors on specific histidine decarboxylase differs in certain important respects from their effect on non-specific histidine decarboxylase. In particular, the specific enzyme, unlike the non-specific enzyme, is scarcely affected by α-methyl-DOPA[40,41,141–143]. Conversely, the specific enzyme is subject to moderate inhibition by α-methylhistidine at concentrations which

have no effect on the non-specific decarboxylase; there is evidence that this inhibition is competitive[8].

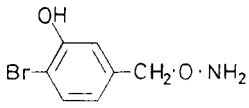
The effect of incorporating the α-methyl group of α-methylhistidine into a cyclopropane ring has been investigated[246]. The resulting compound,



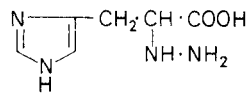
(*XXII*) NSD 1024



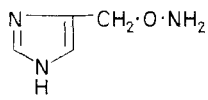
(*XXV*) NSD 1034



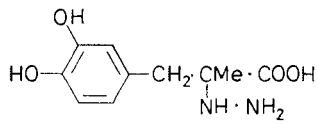
(*XXIII*) NSD 1055



(*XXVI*) MK 785

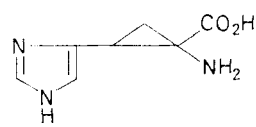


(*XXIV*)
O-(Imidazol-4(5)-ylmethyl) hydroxylamine



(*XXVII*) MK 485

1-amino-2-(4-imidazolyl)-cyclopropane-1-carboxylic acid (*XXVIII*), was found to inhibit the specific histidine decarboxylase of the foetal rat liver to the extent of about 30 per cent at a concentration of 10^{-3}M, i.e. the inhibitory potency is of the same order as that of α-methylhistidine itself.



(*XXVIII*)

The abilities of various compounds to inhibit the specific histidine decarboxylase of rat hepatoma and the non-specific enzyme of guinea-pig kidney have been compared[79,143,144]. Some of these results are given in *Table 4.8*. Of the α-methylamino acids tested, DL-α-methyl-5-HTP was the most potent inhibitor of the specific enzyme. Hydrazine was a strong inhibitor, and the various hydrazides were moderately effective. The *O*-substituted hydroxylamines (*XXII*, *XXIII*, and *XXIV*) and the substituted hydrazine (*XXV*), which we have seen to be potent inhibitors of non-specific histidine decarboxylase, were similarly effective inhibitors of the specific enzyme. The potencies of these compounds may be due, at least in part, to their ability to react with pyridoxal phosphate.

Compounds MK 785 (*XXVI*) and MK 485 (*XXVII*), the α-hydrazino analogues of histidine and α-methyl-DOPA respectively, have been compared as inhibitors of the specific decarboxylase from foetal rats and the

non-specific decarboxylase from guinea-pig kidneys[145]. MK 785 resembles α-methylhistidine in being one of the few compounds known which inhibit specific histidine decarboxylase more strongly than the non-specific enzyme. As it is, in fact, more potent than α-methylhistidine against both enzymes, its effectiveness may be partly due to its acting as a carbonyl reagent, the hydrazino-group combining with pyridoxal phosphate. This point requires further study since, if MK 785 were found to inhibit other pyridoxal-phosphate-dependent enzymes, including in particular histaminase, its value as a tool for elucidating the biological function of specific histidine decarboxylase would be limited owing to the difficulty of interpreting experiments designed for this purpose. MK 485 too may act partly as a carbonyl reagent and partly by interaction with apo-enzyme; this would explain why it is a stronger inhibitor of both histidine decarboxylases than is α-methyl-DOPA, although, like α-methyl-DOPA, it is more effective against the non-specific enzyme.

Inhibition by Inactivation or Displacement of the Co-enzyme

The function of pyridoxal phosphate as the co-enzyme of histidine decarboxylase depends on the ability of the aldehyde group to react with the α-amino group of histidine. However, the aldehyde group also reacts with a wide variety of amino acids, amines and carbonyl reagents. The specificity of the holo-enzyme-substrate interaction is therefore due to the apo-enzyme. The co-enzyme attached to the active centre is presumably in equilibrium with free co-enzyme in the surrounding solution.

The equilibrium constant and the individual rate constants for the formation of Schiff bases between amino compounds and pyridoxal phosphate can be measured spectrophotometrically[122,123]. If two or more amino compounds are present in the solution, then they will compete for the aldehyde group of pyridoxal phosphate, the final equilibrium concentrations of the Schiff bases formed depending on the equilibrium constants[135]. It has already been shown that the formation of new Schiff bases can proceed by direct attack of an amino compound on an already-existing Schiff base, and that the rate of such a reaction may be even faster than with free pyridoxal phosphate[134,135].

When the co-enzyme is attached to the active centre of the enzyme, the rates of formation and breakdown of Schiff bases may differ from those observed with free co-enzyme. This is because the co-enzyme may already be present as a Schiff base at the active centre of the enzyme (*Figure 4.4a*)[107], and also because interaction of the amino compound with the apo-enzyme may alter the rate constants. Inhibition of pyridoxal phosphate-dependent enzymes by reagents such as hydroxylamine or semicarbazide depends on the high affinities of these reagents for the aldehyde group of the co-enzyme. However, as Schiff base formation is readily reversible, this type of inhibition is easily reversed also.

Further reactions may occur after the formation of a Schiff base. Thus aldehydes react with substituted phenylethylamines under mild experimental conditions to form ultimately tetrahydroisoquinolines[146,147]. A particular case of this is the reaction between pyridoxal and DOPA (*Figure 4.5*). A similar reaction occurs between pyridoxal and histidine (*Figure 4.5*). The observation that DOPA decarboxylase is subject to substrate inhibition aroused

further interest in these reactions, and led to kinetic studies of the interaction between pyridoxal phosphate and compounds related either to DOPA or to histidine[51,148]. Typical results are given in *Table 4.9*. 5-HTP and 5-HT also

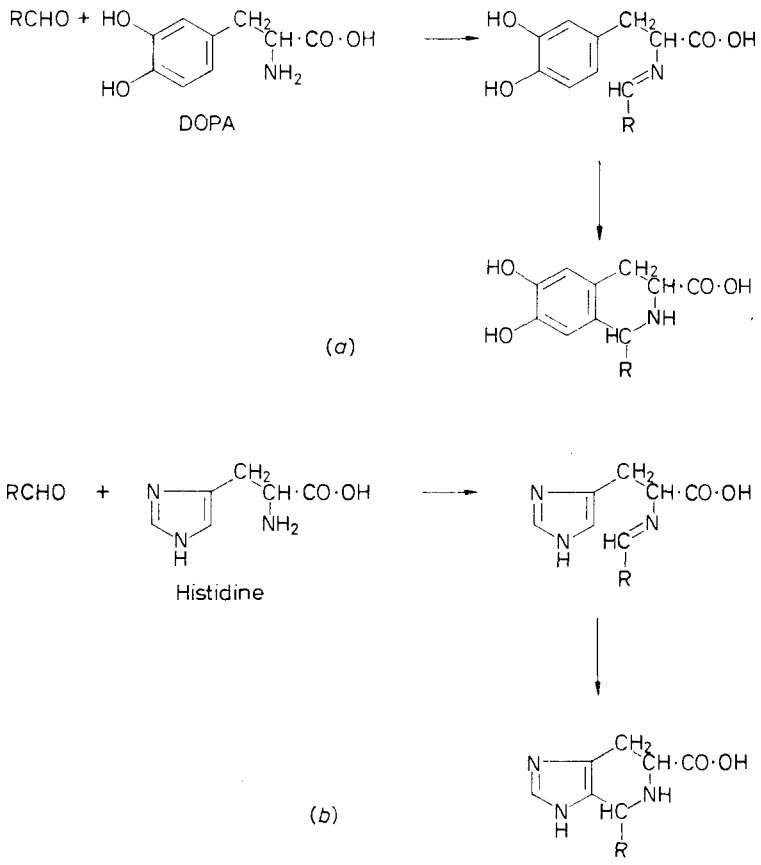


Figure 4.5. Inactivation of pyridoxal phosphate, RCHO: (*a*) by DOPA; and (*b*) by histidine

inactivate pyridoxal phosphate, though less rapidly than do DOPA and dopamine.

Studies of this type indicate that substituted phenylethylamines are effective inactivators of pyridoxal phosphate if they have a phenolic group in the position *meta* to the side chain[51,148–150]. The presence of a methyl substituent in the *alpha* position relative to the amino group markedly slows the rate of inactivation of the co-enzyme[51,143,148].

Studies on the ability of several substituted histidines to inactivate pyridoxal phosphate showed that the presence of electron-donating groups in the imidazole ring increased the rate of reaction. From the values of the appropriate rate constants (*Table 4.10*), it will be seen that 2-thiohistidine, at pH 7·8 and 6·8, inactivates the co-enzyme even more rapidly than does DOPA[143].

Cysteine was also an effective inactivator *in vitro*; the closely related compound penicillamine (β,β-dimethylcysteine) is slightly more reactive[135]. Penicillamine inhibits the transaminases of rat liver[151] and the glutamic acid decarboxylase of mouse brain[152]. The formation of thiazolidine derivatives by reaction of cysteine or penicillamine with pyridoxal phosphate proceeds

Table 4.9. Rates of inactivation of pyridoxal phosphate by various compounds
All experiments were carried out at pH 6·8. k is the second-order rate constant for ring closure (see *Figure 4.5*).

Compound	*Reference*	*Temp.* °C	k(1/mole/sec)
3,4-DOPA	51	23	0·830
3,4-dopamine	51	23	0·238
2,5-DOPA	51	23	0·024
2,4-DOPA	51	23	No reaction
α-methyl-DOPA	51	23	0·057
α-methyl-*m*-tyrosine	51	23	0·068
3-methoxytyrosine	51	23	No reaction
3,4-DOPA	148	38	3·2
Adrenaline	148	38	4·8
m-hydroxypropadrine	148	38	3·2
o-tyrosine	148	38	0·008
m-tyrosine	148	38	2·2
Histamine	148	38	Approx. 2
Histidine	148	38	0·12

so nearly to completion that inhibition of pyridoxal phosphate-dependent enzymes results. However, since the inactivation of pyridoxal phosphate by cysteine and related compounds is reversible, the ability of these substances to inhibit pyridoxal phosphate-dependent enzymes may vary with the affinity of the enzyme for the co-enzyme.

Table 4.10. Rates of inactivation of pyridoxal phosphate by various compounds[143]
All experiments were at 21° ± 4°C. k is the second-order rate constant for ring closure.

Compound	k(1/mole/sec)	
	pH 6·8	pH 7·8
2-thiohistidine	0·73	3·2
2-methylhistidine	0·035	0·10
Histidine	0·006	0·015
3-methylhistidine	0·005	0·005
3,4-DOPA	0·47	0·95
α-methyl-DOPA	0·030	0·050
Cysteine	0·77	1·1

In general, an inactivator of pyridoxal phosphate may attack the free co-enzyme or one of its Schiff bases in the solution. Alternatively the reaction with co-enzyme or with a Schiff base of the co-enzyme may take place at the active centre of the apo-enzyme. Inhibition of pyridoxal phosphate-dependent enzymes by compounds such as DOPA, 2-thiohistidine, or penicillamine, is usually progressive and proceeds much more slowly than the reaction

of these substances with free co-enzyme[143]. This suggests that the rate of removal of co-enzyme from the active centre is rate-limiting, or that the inactivator is slowly attacking the co-enzyme at the active centre. At least part of the observed inhibition could be due to displacement of co-enzyme from the active centre by the pyridoxal phosphate–inactivator complex. More detailed kinetic analyses, preferably utilizing highly purified histidine decarboxylase preparations, will be required before a full picture of this type of inhibition is obtained.

The specific histidine decarboxylases of rat glandular stomach and of foetal rat liver are inhibited by certain acidic anti-inflammatory drugs. The inhibition is non-competitive with respect to substrate, but is competitive with respect to co-enzyme. The specific enzyme has a relatively low affinity for pyridoxal phosphate which it probably binds by means of the ε-amino group of a lysyl side-chain. There is evidence that acidic anti-inflammatory drugs produce their inhibition by displacing pyridoxal phosphate from this ε-aminolysyl group of the apo-enzyme[223,224,255]. Non-specific histidine decarboxylase which has a greater affinity for pyridoxal phosphate is not inhibited by these drugs in this way.

INHIBITION OF HISTIDINE DECARBOXYLATION *in vivo*

The *in vivo* production of histamine, and of other amines, in rats can be diminished by the use of pyridoxine-deficient diets, pyridoxine antagonists, or non-specific inhibitors such as semicarbazide[111,154,155,156]. In female rats receiving a pyridoxine-deficient, histamine-free diet, the urinary output of histamine was reduced to about 50% of normal[157]; simultaneous administration of semicarbazide further reduced the histamine output to about 20% of normal. However, the histamine content of the tissues of these animals did not differ significantly from those of controls, except in the stomach where the histamine content dropped to a few per cent of normal.

The use of a more specific inhibitor of histidine decarboxylase was obviously desirable, and such experiments were carried out with α-methylhistidine[158]. Administration of this compound to female rats maintained on a standard histamine-free diet, decreased the histamine output to less than 50 per cent of the normal value. When this treatment was superimposed on pyridoxine deficiency, the urinary histamine was reduced to 25 per cent of normal. α-Methylhistidine produced no detectable change in the urinary output of 5-hydroxyindolyl-3-acetic acid (5-HIAA), the metabolite of 5-hydroxytryptamine (5-HT); this suggests that only the specific histidine decarboxylase was being inhibited. In agreement with this view it has been found that α-methyl-DOPA, which selectively inhibits the non-specific histidine decarboxylase, does not lower urinary histamine excretion in the rat[145,243]. α-Methyl-DOPA does not diminish the basal excretion of histamine by the guinea-pig[253]; however, it decreases considerably the output of histamine in the urine of guinea-pigs after feeding of histidine[253], and the excretion of ^{14}C-histamine following the injection of ^{14}C-histidine[159,225]. Thus it appears that the non-specific enzyme of the guinea-pig is active *in vivo*, but some contribution by the specific enzyme is not excluded. This point requires further elucidation.

Compound NSD 1055 (*XXIII*) and MK 785, the α-hydrazino analogue of

histidine (*XXVI*), inhibit the specific histidine decarboxylase of the foetal rat, while NSD 1055, α-methyl-DOPA and MK 485, the α-hydrazino analogue of α-methyl-DOPA (*XXVII*), inhibit the non-specific decarboxylase of guinea-pig kidney[145]. However, when administered to female rats, only NSD 1055 and MK 785 produced any appreciable effect on the histamine content of the tissues; they produced lower levels of histamine in the heart, stomach and urine, but had no effect on the histamine content of the peritoneal mast cells. Administration of these two inhibitors to rats pretreated with compound 48/80, did not produce any further reduction in the histamine content of the tissues. Whereas regular administration of NSD 1055 killed the rats within several days, MK 785 appeared to be non-toxic. This compound produced its maximum effect within 3–6 hours, and the tissue histamine content had returned to normal within 24 hours. NSD 1055 has also been reported to inhibit histamine biosynthesis in man[257]. Although α-methyl-5-HTP has also been shown to be an efficient inhibitor of specific histidine decarboxylase *in vitro*[79], it neither lowered the histamine content of the organs nor modified the anaphylactic response in guinea-pigs[160]. Quercitin, a flavone derivative, has been reported to be active *in vitro* and *in vivo* as an inhibitor of specific and non-specific histidine decarboxylase derived from rat and mouse tissues[161].

The results obtained with inhibitors suggest that it is a specific histidine decarboxylase which is mainly involved in the production of histamine in the rat. Studies on the excretion of histamine in the urine of thyroxine-treated[83,164–166] and of totally gastrectomized[83] rats indicate that the specific histidine decarboxylase responsible for this histamine production is located mainly in the mucosa of the glandular portion of the stomach.

There is considerable doubt at present concerning the physiological significance of the non-specific histidine decarboxylase[76,145]. Nevertheless, the possibility remains that some of the compounds which have been found to inhibit the formation of dopamine and 5-HT may also be useful inhibitors of histamine formation. Comparative potencies, *in vitro* and *in vivo*, of various substances as inhibitors of the non-specific decarboxylase with DOPA as substrate have been recorded in the literature[162,163].

FACTORS WHICH MODIFY THE HISTIDINE DECARBOXYLASE ACTIVITY OF TISSUES

The histidine decarboxylase activity of tissues can be raised or lowered by changing the hormonal state of the animal or by subjecting it to certain stressful stimuli. Administration of thyroid hormones to rats produces a marked increase in the specific histidine decarboxylase activity of the glandular mucosa of the stomach[83,164–166], while the activity of the non-specific enzyme in the liver is lowered[167]. Studies of the action of thyroid hormones on other pyridoxal-dependent enzymes in rat liver suggest that, in this organ at least, these changes arise from corresponding alterations in both pyridoxal phosphate and apo-enzyme synthesis[247].

The histidine decarboxylase activity of rat lung is considerably depressed by the administration of glucocorticoids[168,169] whereas the activity in the lung of mice or guinea-pigs is virtually unchanged[168]. In the rat, treatment with

glucocorticoids promotes increased formation of histamine in the glandular mucosa of the stomach and diminishes the activity in the liver[76,170]. Compounds having the highest glucocorticoid potency produced the greatest changes in histidine decarboxylase activities[170]. The 5-HTP decarboxylase activity of rat liver is said to be unaffected by glucocorticoid treatment. This might seem to indicate that 5-HTP decarboxylase is not after all identical with non-specific histidine decarboxylase. It is known, however, that hormonal changes can alter the pyridoxal phosphate content of tissues[211], and that the decarboxylation of histidine is more sensitive than that of 5-HTP to changes in the concentration of the co-enzyme[59]. Further studies over a range of concentrations of the co-enzyme are required to elucidate this point. Adrenalectomy in rats usually has effects opposite to those produced by the injection of glucocorticoids[171]. (See note on page 245.)

Administration of the histamine liberators, compound 48/80 and polymyxin B, or exposure to cold, produces, on the liver and glandular portion of the rat stomach, effects similar to those of glucocorticoids[170]. Indeed, the effects may be mediated by endogenous glucocorticoids released as a result of the severe stress evoked by these agents. Several studies of the influence of various stressful stimuli, local and systemic, on the histidine decarboxylase activity of tissues have been carried out. Stimuli such as the injection of catecholamines or bacterial endotoxins, anaphylaxis, exposure to cold, exercise and anoxia and the application of tourniquet shock produce an increase in the histidine decarboxylase activity of some tissues of rats, mice, guinea-pigs and cats[84,169,172–174,258]. Detailed studies in the mouse show that injections of adrenaline increase the histidine decarboxylase activity of skin, lung and skeletal muscle, and that these increases are not prevented by prior administration of α- or β-adrenergic blocking agents[100,169,172]. Occasionally, stress leads to a diminution of histidine decarboxylase activity; this has been observed in certain species after exposure to warmth[76], and in the kidneys of rabbits which have been treated with bacterial endotoxins[175]. This enzyme, which undergoes changes in activity in response to stressful stimuli, has been called inducible histidine decarboxylase by Schayer; its possible function will be considered later.

THE PHYSIOLOGICAL FUNCTION OF HISTIDINE DECARBOXYLASES

Merely to state that the physiological function of the histidine decarboxylases is the conversion of histidine to histamine is to evade the problem of the function of histamine itself. A compromise will therefore be adopted so that due account can be taken of the ways in which the search for possible roles for histamine in physiological processes has shed light on the function of the enzymes by which histamine is formed.

The first question to arise is whether the two main classes of mammalian histidine decarboxylase, the specific and non-specific enzymes, are of equal importance in relation to the physiological function of histamine. Consideration of the values of the Michaelis constant, approximately 10^{-3}M for the specific, and 10^{-1}M for the non-specific, enzyme (*Table 4.6*), indicates that the specific histidine decarboxylase has the greater affinity for histidine. This might be taken to imply that the specific enzyme is the more important source of histamine in the body[220].

Such considerations do not, however, exclude the participation of the non-specific enzyme as a source of body histamine. The non-specific histidine decarboxylase of guinea-pig kidney is known to have a high affinity for DOPA and 5-HTP, but a low affinity for histidine and phenylalanine[46]. At first sight, then, it would appear that this enzyme is more likely to produce dopamine and 5-hydroxytryptamine than to form histamine or β-phenylethylamine. It must be remembered, however, that the substrates DOPA and 5-HTP are not normally detectable in blood or tissues, while histidine and phenylalanine are present in amounts which compensate for the low affinity of the enzyme for these two amino acids. In terms of the capacity to form the corresponding amines, therefore, there is no reason to suppose that the decarboxylation of histidine is a less important function of the non-specific enzyme than is the decarboxylation of its other substrates.

Consideration must also be given to the possible role of bacterial histidine decarboxylase as a source of tissue histamine. In cat, dog and man, for example, histidine decarboxylase is either absent or occurs in amounts which seem too small to account for all the histamine present in the tissues (*Table 4.6*). It is known[37] that histamine can be absorbed from the intestine of dogs and humans, and that oral administration of histidine in dog, man and guinea-pig results in a rise in urinary free histamine. If the intestinal tract is sterilized prior to the administration of histidine, however, the rise in urinary histamine is prevented in dog and man, but still occurs in guinea-pigs[254]. Since some tissues of guinea-pigs and other rodents have considerable histidine decarboxylase activity, it is possible that these species synthesize most, or all, of their own histamine, while cat, dog and man, which possess only trace amounts of the enzyme, may obtain their tissue histamine largely by absorbing it from the gut where it has been formed by the bacterial decarboxylation of dietary histidine.

Nevertheless, when factors which modify the histidine decarboxylase activity of tissues are considered, it is found that the most striking changes occur in the level of the specific enzyme. We have already seen that this is true of the effect of inhibitors and of certain external factors, but it should be noted that most of this information has been derived from studies in the rat. Various physiological and pathological conditions are now described which also lead to changes in the activity of the specific histidine decarboxylase in the tissues of rats and other animal species.

Histidine Decarboxylase and Growth

There is evidence that a greatly increased histidine decarboxylase activity is associated with certain types of rapid tissue growth[178,179]. Thus, pregnant rats excrete abnormally large amounts of histamine in the urine from about the 15th day of gestation until term, and this increased histamine formation has been ascribed to the developing foetuses. All tissues of the rat foetus have higher histamine-forming capacities than the corresponding tissues of the normal adult. The foetal liver is by far the most active of these, yet within 2–3 days after birth its activity falls precipitously to the low level observed in adult rats[180]. The histidine decarboxylase of the foetal rat liver has been fully characterized (*Table 4.4*), and its properties clearly indicate that it is the specific enzyme. The feeding of a pyridoxine-deficient diet and the

simultaneous injection of semicarbazide arrested the growth of the foetuses and led to their death. It is possible, therefore, that this failure of embryonic development was due to the inhibition of histidine decarboxylase, and that the enzyme is essential to the growth process. However, the above treatment is likely to lead to varying degrees of inhibition of all pyridoxal-dependent enzymes, and the observed effect may, therefore, be due, at least in part, to the inhibition of one of these other enzymes. Unfortunately, no inhibitor of the histidine decarboxylase of foetal rat liver is known which is sufficiently potent and specific to be used in the above experiment, and thus to place the matter beyond doubt.

Adult rats in which the liver is regenerating after partial hepatectomy excrete increased amounts of histamine in the urine, and this has been attributed to the growth of the regenerating liver[178]. However, the histamine does not originate in the liver, as no increased histidine decarboxylase activity is demonstrable in the regenerating tissue *in vitro*[226,227].

The peak of histidine decarboxylase activity in foetal rat liver coincides approximately with that of haemopoietic activity[181]. Subsequently, the bone marrow of the adult rat was found to contain a specific histidine decarboxylase (*Table 4.4*), thus supporting the possibility of a connection between haemopoiesis and the formation of histamine[182]. Histidine decarboxylase also occurs in the bone marrow of the guinea-pig; this enzyme activity, which was shown to be related to the number of basophils, does not, however, support a relationship between histidine decarboxylase activity and growth, since the basophils in the buffy layer of guinea-pig blood have also a considerable histamine-forming capacity, but contain no cells in mitosis[183].

A connection between histidine decarboxylase activity and rapid tissue growth has also been sought by studying reparative growth of healing skin wounds. In the rat, wound tissue excised 24 hours after the infliction of a wound had 50–60 times more histidine decarboxylase activity than control skin. Moreover, when the histidine decarboxylase activity of the skin, prior to wounding, was artificially lowered by cortisone treatment[184,185] or by subjecting the animals to simultaneous pyridoxine deficiency and administration of semicarbazide[179], the rate of healing was greatly retarded. Conversely, when the specific histidine decarboxylase activity of the skin was increased by repeated injection of the histamine liberators 48/80 or polymyxin B[186], the rate of healing was significantly accelerated[179]. Other workers, however, have reported either no effect on[187], or actual retardation of[188], wound healing in rat skin after compound 48/80 treatment. There is some evidence that wound tissue in human skin, particularly in the early stages of healing, has a higher histidine decarboxylase activity than normal skin[189,190]. Further studies are required to substantiate this, however. Care must be exercised in the interpretation of these experiments relating histidine decarboxylase activity to the healing of skin wounds, as some of the histamine formed may arise from mast cells. Moreover, the agents employed for artificial raising or lowering of histamine formation in the skin possess other physiological effects which may modify the healing process irrespective of the action on histidine decarboxylase activity.

The formation of collagen also provides an index of reparative growth. In agreement with the view that there is a relationship between histidine

decarboxylase activity and growth it has been found that when the histidine decarboxylase level in rat skin has been artificially raised, as in the experiments reported above, the rate of collagen formation in healing wounds is increased; conversely, when the histidine decarboxylase level is lowered, the rate of collagen formation is diminished[184,191,192]. When the rats, prior to wounding, were subjected to prolonged, high tissue histamine levels from subcutaneously-implanted histamine dipicrate, neither healing nor collagen formation was accelerated. This indicates that if histamine is indeed involved in wound healing it must be formed, and must act, intracellularly.

Histidine Decarboxylase and Tumour Growth

The recognition of a possible connection between histidine decarboxylase activity and growth then raised the question whether a high histidine decarboxylase activity might be an important factor in rapidly-growing, malignant tissues. Studies in August strain rats bearing subcutaneously-implanted hepatoma tissue (F-Hep) indicated that this might be so. Soon after implantation of the tumour the urinary histamine excretion in these rats increased dramatically, but returned to normal immediately after removal of the tumour[193]. The tumour itself possessed high histidine decarboxylase activity which, on further investigation, proved to be due to the specific enzyme (*Table 4.4*)[39]. Similar observations have been reported in rats bearing a virus-induced sarcoma; in these animals the liver was found to be enlarged and to have considerably increased HFC. Studies with inhibitors indicated that the histidine decarboxylase in the tumour and in the liver of the tumour-bearing rats was the specific enzyme[249]. However, experiments with other subcutaneously-transplanted tumours in the rat have failed to demonstrate any relationship between histidine decarboxylase activity and tumour growth[80,227].

Conflicting results have been reported with the Walker rat carcinosarcoma 256. The extremely small histidine decarboxylase activity of this tissue at pH 6·5 has been regarded by Håkanson[194] as indicating a relationship between the enzyme activity and rapid tissue growth, while other workers[80,195] take the opposite view. The disagreement is due in part to methodology, Håkanson having used the highly sensitive isotopic procedure while the others employed the biological method. Nevertheless, the activity of the Walker tumour as measured isotopically is exceedingly small in comparison with the activities obtained by the same method for tissues such as foetal rat liver and rat bone marrow. It is doubtful, therefore, whether this low activity should be taken as support for a relationship between histidine decarboxylase activity and growth. In the light of subsequent studies Håkanson[250] considers that an active biosynthesis of histamine may be a common phenomenon in experimental tumours of the rat and mouse, but he does not regard a high HFC as a necessary feature of tumour growth, since no histidine decarboxylase activity could be detected in a hamster melanoma.

A hamster hepatoma was likewise found to be devoid of histidine decarboxylase activity[80].

It has been seen that a specific histidine decarboxylase is present in normal mast cells; however, since mitoses are never observed in these cells, it is unlikely that the presence of the enzyme is concerned with growth. Moreover,

of two mast cell tumours in mice, one, the Furth mastocytoma grows slowly, but has high histidine decarboxylase activity, whereas the other, the Dunn P-815 mastocytoma, grows very rapidly, and has small, or sometimes no, histidine decarboxylase activity[196]. These observations do not support the theory of a connection between histidine decarboxylase activity and the rate of growth.

The histidine decarboxylase activity in rat liver has been followed throughout the period of carcinogenesis in animals treated with the hepatocarcinogen diethylnitrosamine (DENA)[57]. The normal liver was devoid of the specific enzyme but contained non-specific enzyme, the activity of which decreased after the animal had received about one month's treatment with DENA. Later, specific histidine decarboxylase was detected, its formation coinciding with the appearance of hepatomatous nodules in the liver parenchyma. Further treatment with DENA led to a vigorous proliferation of bile duct cells, and this cholangiomatous condition was accompanied by a steep decrease in the specific histidine decarboxylase activity. It was concluded, therefore, that specific histidine decarboxylase may be associated with the growth of a particular type of cell, such as the hepatoma cell, but not with growth in general.

Kahlson, Rosengren and Steinhardt[197] in studies of the Landschutz I tumour growing as isolated, free cells in the mouse peritoneal cavity, have found that on the first day of tumour growth the specific histidine decarboxylase activity is high and correlates with the frequency of tumour cell mitosis. However, it is possible that some of this enzyme activity may have been contributed by the peritoneal mast cells of the host.

Attempts to arrest the growth of histidine decarboxylase-containing tumours by histidine decarboxylase inhibitors have been unsuccessful. Semicarbazide produced some inhibition of the growth of a subcutaneously-transplanted hepatoma in Wistar rats[80], but in view of the high doses required and the lack of specificity of this compound, the effect was not necessarily due to the inhibition of histidine decarboxylase. The extremely potent hydrazine and hydroxylamine derivatives, NSD 1055 (*XXIII*), and NSD 1034 (*XXV*) failed to prevent the growth of subcutaneous transplants of the hepatoma, F-Hep, in August strain rats[198]. Transplants of this tumour in pyridoxine-deficient August rats grew more slowly than in normal controls, but the animals did not survive long enough to yield conclusive results. Attempts to inhibit the development of the Landschutz I tumour growing in the mouse peritoneal cavity were also inconclusive[197]. All the above experiments are difficult to interpret, not only for the reasons already given, but because it is unlikely that total inhibition of the histidine decarboxylase would be obtained[8,25]. It is by no means uncommon for an enzyme to be present greatly in excess of the body's needs, and histidine decarboxylase could conceivably discharge its physiological function satisfactorily even when inhibited to the extent of 90 per cent or more.

Histidine Decarboxylase and Pregnancy

We have seen that the first indication of a relationship between histidine decarboxylase and growth arose from studies in pregnant rats. Increased specific histidine decarboxylase activity was found in the foetal liver and the

maternal stomach[176,180]. Only in the mouse and hamster have comparable changes in histidine decarboxylase activity been detected during pregnancy. In both species, as in the rat, this leads to a greatly increased excretion of histamine in the urine. In the mouse the histidine decarboxylase activity of the whole foetus increased steadily throughout pregnancy, but fell sharply at birth; this activity was located mainly in the foetal skin[87,88]. At the same time the kidney of the mother showed a greatly increased activity compared with that in the kidney of normal females, and this high activity persisted for a few weeks after delivery. The enzyme of both the foetal skin and maternal kidney was the specific histidine decarboxylase. In the pregnant hamster the urinary histamine excretion was particularly high during the last three days of gestation, but returned almost to normal on the first day after delivery. The large histamine output was traced to a very high histidine decarboxylase activity in the placenta[102]. This enzyme has not yet been fully characterized. No indication of increased histidine decarboxylase activity has been found in foetal, as compared with adult, tissues of the hamster, rabbit, ferret, pig, cow[98], cat[98,199] or guinea-pig[98,199,228]. Relatively little is known about histidine decarboxylase in human pregnancy owing to the limited availability of experimental material. In clinically normal human pregnancies, the urinary histamine excretion is either unchanged[200,201] or only slightly raised[202]. Human foetal tissues contain only traces of a non-specific histidine decarboxylase, and the amounts do not appear to exceed those of human adult tissues[103,203].

Histidine Decarboxylase and Gastric Secretion

Histamine is normally present in the stomach mucosa of mammals, and the gastric juice always contains histamine[204], though only in small amounts[229]. The question thus arises whether this histamine is produced by the local decarboxylation of histidine. The histamine-forming capacity (HFC) of the gastric mucosa would not by itself be a reliable guide in deciding this point, as we have already seen that there is no exact correlation between the histamine content and the histidine decarboxylase activity of tissues. It is known, however, that in species which possess a very high mucosal HFC, the sensitivity of the acid-secreting cells to injected histamine is very low, and that, conversely, where mucosal HFC is low the acid-secreting cells are extremely sensitive to injected histamine[177]. Thus the histidine decarboxylase activity in the gastric mucosa of all species studied is sufficient to produce the amounts of histamine necessary to stimulate acid secretion. Does histidine decarboxylase, then, have a physiological role in relation to gastric acid secretion?

There is some evidence that this is so. In the rat, the release of endogenous gastrin on feeding liberates histamine in amounts sufficient to stimulate gastric acid secretion[30]. Studies with exogenous gastrin in the rat support this view[205]. When rats are fed normally or when they are injected with gastrin, acid secretion occurs[177] accompanied by an increased output of urinary histamine[177], a decrease in the histamine content of the gastric mucosa[177] and an increase in the histidine decarboxylase activity[177,226,230]. The increased mucosal HFC persists for some time after acid secretion has ceased, indicating that the depleted store of mucosal histamine is then being replenished. Thus a feed-back mechanism may exist, and this possibility is

supported by the observation that the enzyme activity is lowered when acid secretion is evoked by the injection of exogenous histamine.

There is no significant reduction in the histamine content of the gastric mucosa of cat, dog or guinea-pig after feeding. In these species the histamine stores must be replenished during acid secretion, although the HFC of their gastric mucosa seems inadequate to produce the amount of histamine found there. It is possible, therefore, that the histamine in the gastric mucosa of these species is formed elsewhere and transported by the blood stream to the

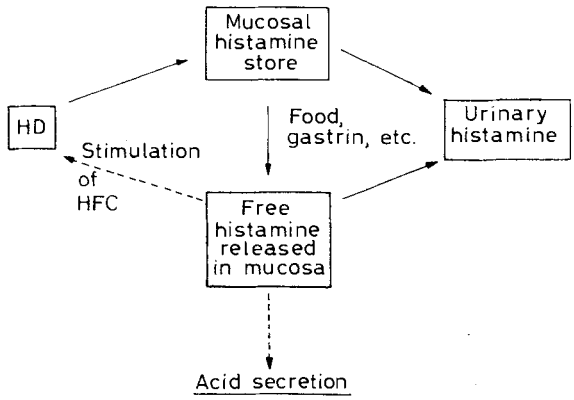


Figure 4.6. Possible role of histidine decarboxylase in gastric secretion

mucosa where it is then bound[229]. Although the HFC of mouse stomach has not yet been the subject of such detailed study as that of rat stomach, it is greatly stimulated by feeding, being about 100 times greater in normally-fed mice than in starved animals[100]. Thus there is evidence, at least in the rat and the mouse, that the function of the histidine decarboxylase of the stomach is to produce the histamine required to stimulate gastric secretion.

Further evidence for a role of histidine decarboxylase in relation to gastric secretion has been obtained in rats treated with histidine decarboxylase inhibitors. Pretreatment of rats with MK 785 (*XXVI*) or with NSD 1055 (*XXIII*) produces a period of maximum depletion of histamine in the gastric mucosa during which basal acid secretion is diminished and stimulation of acid secretion by gastrin and other agents is significantly blocked[231]. Similarly the gastric hypersecretion which results in rats following the formation of a portacaval shunt is inhibited by administration of NSD 1055[232]. In pyridoxine-deficient rats the resulting decrease in the HFC of the stomach is associated with a marked reduction in gastric secretory activity[233]. On the other hand, Kahlson and co-workers[177] found that the histamine content and the HFC of rat gastric mucosa could be reduced to only 5–10 per cent of normal by administration of semicarbazide and a pyridoxine-deficient diet, and yet acid secretion in response to the injection of gastrin occurred as in normal rats. The divergence of this observation from those of other workers may be

due to the use of different techniques for the measurement of gastric secretion[231,234].

It appears, then, that the role of histidine decarboxylase in relation to gastric secretion is to replenish the store of mucosal histamine which is released in response to feeding, there being a temporary adaptive increase in enzyme activity until the histamine store is replenished. These relationships are shown diagrammatically in *Figure 4.6.*

Inducible Histidine Decarboxylase

The histidine decarboxylase which is formed in several animal species under various stressful stimuli has the characteristics of a specific histidine decarboxylase (*Table 4.4*)[76]. The evidence available at present indicates that the inducible enzyme is not associated with mast cells[76,207]. Schayer has suggested that this enzyme is located in or near vascular endothelial cells, and that it synthesizes histamine at a rate which is determined by the needs of the tissues for blood under given environmental conditions. The role of inducible histidine decarboxylase may thus be to produce histamine as required for the maintenance of circulatory homeostasis[84,174,248]. Schayer postulates that the progressive microcirculatory dilatation occurring in slowly-developing inflammation and in systemic stress is due to enhanced formation of histamine arising from adaptively increased activity of the inducible histidine decarboxylase. Circulatory homeostasis is considered to involve an antagonism between histamine formed in this way and circulating glucocorticoids[235,236,237].

Activation of the inducible enzyme becomes detectable 0·5–1 hour after application of the stress, and it persists for periods which depend on the nature and intensity of the stimulus. Under exceptional circumstances the high rate of induced histamine formatation may be sufficiently prolonged to result in the development of shock[84]. On the other hand, when the degree of activation of histidine decarboxylase is inadequate, tissue damage may occur as has been observed in the kidneys of endotoxin-treated rabbits[175]. If histamine produced by inducible histidine decarboxylase is indeed a mediator of the slow phase of inflammation[235], inhibitors of this enzyme might possess anti-inflammatory action. It is thus of considerable interest that the ability of one class of anti-inflammatory drugs, the acidic group, to inhibit specific histidine decarboxylase runs parallel to their clinical activity[223,255].

CONCLUDING REMARKS

The above considerations emphasize that the rate at which histamine is formed by a tissue is of greater significance than the amount of histamine actually present in that tissue. Thus, the determination of histidine decarboxylase activity represents a dynamic approach to the elucidation of the physiological function of histamine, in contrast to the earlier static approach based on measurements of the histamine content of tissues[96,238].

Histidine decarboxylase may have a physiological role in relation to normal and certain types of pathological growth, to wound healing, pregnancy, gastric secretion, inflammation and the reaction to stressful stimuli. These are situations in which an adequate local blood supply is important. This blood supply may be regulated through histamine produced by a

substrate-specific histidine decarboxylase developed for the purpose. The histamine is formed within the cells on which it is to act, and it is therefore 'intrinsic histamine' as envisaged by Dale[208]. This is non-mast cell histamine having a high turnover rate and apparently comprising much of the histamine formed in the body[239]. It is mobilized by cholinergic stimulation, and the suggestion has been made that it functions as the final common stimulant in the formation of exocrine secretions[239]. Most of the information so far available has been derived from experiments in rats. In other species the physiological relationships are less obvious or apparently do not exist[98,199,228]; indeed, discrepancies have been noted in the rat itself[57,240]. Further investigations covering a yet wider range of species are necessary for the full picture to emerge.

The specific histidine decarboxylase of mast cells apparently produces histamine for local storage within the mast cell itself. This may be the primary function of the mast cell[209].

The function of the non-specific histidine decarboxylase of rabbit or guinea-pig liver and kidney remains to be clarified. However, in view of its wide substrate specificity (*Table 4.3*), this enzyme may rather be a general aromatic L-amino acid decarboxylase, the purpose of which is to produce other physiologically important amines in addition to histamine.

Note. The effect of sex hormones on the histidine decarboxylase activity of tissues has not been systematically examined, but the administration of oestradiol to ovariectomized female mice produces a substantial increase in the histamine-forming capacity of the liver[259].

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PSYCHOTROPIC DRUGS AND NEUROHUMORAL SUBSTANCES IN THE CENTRAL NERVOUS SYSTEM

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INTRODUCTION

DURING the past 15 years or so, the problem of the biochemical aetiology of mental illness and of the mode of action of drugs which act on the central nervous system has become the object of intensive study. The almost simultaneous occurrence of several separate events precedented a great deal of research in this field of medicine. Foremost amongst these events was the discovery of whole new ranges of compounds which had a powerful effect on emotional and behavioural responses. Because of their use in the treatment of neurotic and psychotic conditions, these drugs were said to have psychotropic, psychoactive or phenotropic actions. The discovery of the psychotropic properties of the original members of this group of drugs was made during investigations into their activity in a number of conditions not directly related to the central nervous system. In the search for compounds which would combine an intense psychotropic effect with minimal peripheral activity, a large number of derivatives were synthesized, many of which were found to be therapeutically useful. Some measure of the impact of these psychotropic drugs on psychiatry and pharmacology is provided by the estimate that, in the first ten years of their use, just one of them, chlorpromazine, was mentioned in 10,000 publications and was prescribed for 50 million patients throughout the world[1]. During the period of development of the psychotropic drugs, a major advance took place on the physiological front; it became unequivocally established that the transmission of nerve impulses across synapses in the central nervous system is mediated by chemical substances. A nerve impulse, arriving at the end of a nerve terminal, liberates a tiny jet of a chemical mediator which diffuses across the synapse so exciting or inhibiting the next neurone in the functional chain. It also became apparent that there is no universal transmitter substance. The search for other substances stimulated as large a research effort as had the introduction of the new psychotropic drugs. The knowledge that specific chemical substances were involved in the most fundamental of central nervous processes raised hopes that the actions of the psychotropic drugs could be explained in terms of an interaction with the processes involved in the synthesis, storage, release, physiological activity or destruction of the transmitter substances. If this were so, it seemed clear that mental illness itself might have its origin in disturbances of transmitter metabolism. The observation that the activity of 5-hydroxytryptamine, a possible transmitter substance, was inhibited by lysergic acid[2,3], a potent hallucinogen which produces in normal man a state reminiscent of schizophrenia, gave added credence to this view of the origin of mental illness. It also led to studies

which added enormously to the flood of information concerning the relationship between the psychotropic drugs and chemical substances in the brain. 5-Hydroxytryptamine, indeed, has been the subject of as many papers as has chlorpromazine.

In spite of the great effort which has been expended by a vast number of workers throughout the world, only one central transmitter substance—acetylcholine—has been unequivocally identified. Little precise information is yet available concerning either the identity of the other transmitter substances or the mode of action of the psychotropic drugs. The non-specialist who seeks to assess the present situation in this field is likely to be daunted by the sheer mass of information available, discouraged by the conflicting conclusions with which he is presented, and misled by some of the illfounded dogmatisms he will read. It is hoped that this review will provide him with a reasonably objective picture of the present state of knowledge and with the means of assessing for himself the significance of future developments in this area. It is based on the findings reported in several thousand publications (of which only a representative sample can be quoted) and summarizes the more acceptable conclusions concerning the transmitter role of the pharmacologically active substances found in the brain; it also surveys the nature and properties of the more important psychotropic drugs. The more strictly pharmacological portions of the review are preceded by a brief account of some of the principal neurophysiological systems which control the level of consciousness, emotion and muscle tone. All these conditions are likely to be altered in the course of treatment with psychotropic drugs.

THE PHYSIOLOGICAL BACKGROUND

Sensory nerve impulses destined to reach consciousness, travel from the periphery to the brain by well-defined tracts in the spinal cord which come together in the medulla and continue as the lemnisci to the thalamus, whence they are relayed, via the internal capsule, to the sensory cortex (Area 3) in the parietal lobe of the hemisphere. Sensory impulses in the cranial nerves also travel to the thalamus and parietal cortex. (The paths taken by impulses generated in the organs of special sense—the eyes, ears and nose—need not be considered here.) The information transmitted along these routes is interpreted in terms of specific sensations (pain, touch, etc.) well localized in consciousness to the part of the body from which they originate. These ascending impulses, however, simultaneously stimulate another system of neurones. Collaterals from the lemniscal fibres make synaptic contact with a diffuse group of cells—the reticular neurones—in the mid-brain and hypothalamus. From the reticular neurones impulses pass to the frontal, and to a lesser degree, the occipital and temporal lobes. Since they do not end specifically in Area 3 of the cortex, they are not appreciated in consciousness and they have no localizing function. Their function seems to be that of keeping the cortex in an 'alerted' condition, enabling it to respond to the specific signals being carried by the lemniscal pathways. The maintenance of consciousness depends on the continued activity of the ascending reticular system: when its fibres are cut in experimental preparations, the animal exhibits both the behavioural and the electroencephalographic signs of sleep. Stimulation of the reticular neurones

in such animals causes an 'alerting' response with opening of the eyes and restoration of the electroencephalogram to its waking form. Interruption of impulses in the direct sensory pathway has no effect on the level of consciousness. Although impulses travelling to the cortex by the reticular system are not themselves appreciated in consciousness, the conscious appreciation of impulses travelling by the lemniscal route is impossible in the absence of activity in the reticular system.

The multiplicity of synapses in the mid-brain and hypothalamus makes the reticular system particularly susceptible to chemical influences and a large number of drugs are capable of modifying its activity, thus producing changes in the level of consciousness, alertness and behavioural responsiveness.

The reticular system also has a descending component made up of both excitatory and inhibitory fibres which arise in the brain stem, converge on the anterior horn cells of the spinal cord and regulate the tone of skeletal muscle. The descending reticular system is itself subjected to influence from the cerebellum and the basal ganglia and the whole complex can be described as the extrapyramidal system. No two neurophysiologists agree as to the precise constitution of the extrapyramidal system or to its exact relationship with the reticular system, but since the pyramidal system includes only the tracts of fibres which pass from the motor cortex to the anterior horn cells, and carry the impulses subserving voluntary motor activity, it is reasonable to include in the extrapyramidal system all the other descending tracts and their associated nuclei which influence the activity of the anterior horn cells. The integrity of the extrapyramidal system is essential if voluntary motor activity is to be smoothly executed. Disorders of extrapyramidal function cause disturbances of muscle tone and co-ordination.

The basal ganglia are nuclear masses at the base of the brain. A simple enumeration of the components of the basal ganglia is sufficient for the purpose of the present discussion: details of their precise locations and of their connection with one another and with the rest of the brain (insofar as these are at present known) can be found in textbooks of neuroanatomy. The principal basal ganglia are the caudate nucleus, the putamen and the globus pallidus. These three nuclei are collectively described as the corpus striatum; the putamen and globus pallidus are sometimes called the lentiform nucleus. Other structures included in the basal ganglia include the claustrum, the amygdaloid bodies and the substantia nigra.

Lesions of the basal ganglia give rise to a variety of diseases, all of which are characterized by a symptomatology which includes varying degrees of athetosis (involuntary movements of the limbs and facial muscles), rigidity and tremor. The diseases include cerebral palsy, torsion spasm, Huntington's chorea (St. Vitus' dance), hemiballism and two disorders of particular interest to the neuropharmacologist—Wilson's disease and Parkinson's disease. Wilson's disease (hepato-lenticular degeneration) results from an inborn error of metabolism which deposits protein-bound copper in the liver and brain, particularly the putamen and thalamus. Large amounts of free amino acids are excreted in the urine. Extrapyramidal symptoms and intellectual deterioration are severe. In Parkinson's disease (paralysis agitans) the muscles are rigid and the tremor is intensified during emotional stress.

It may take the form of a pill-rolling movement of the thumb and index finger. The facial muscles are immobile and the gait is festinant.

The neurones of the extrapyramidal system are drug-sensitive and extrapyramidal effects ranging from slight rigidity to a fully-blown Parkinson's syndrome are frequently seen during psychotropic drug therapy. The existence of many different forms of extrapyramidal disease is a manifestation of the complexity of the basal ganglia and their interconnections. It is not yet possible to ascribe individual diseases to lesions of specific components of the basal ganglia. Many workers, following the observation of Tretiakoff[4] in the early years of the century, have asserted that pathological changes in substantia nigra are always seen in Parkinson's disease[5] but the careful analyses of Denny-Brown[6] have shown that this is not so. Experimentally placed lesions of the substantia nigra in monkeys do not cause the appearance of Parkinsonism.

The hypothalamus is also an important component of the neural system whose activity determines the emotional state. Emotion is a way of feeling and a way of behaving; the autonomic nervous system, whose activity is controlled from the hypothalamus, is involved in both these aspects of emotional change. Impulses from the viscera play some part in determining the affective aspect of emotion and changed activity in autonomically innervated structures (the blood vessels, the heart and the intestines, for instance) is a characteristic sign of emotional tension. Observations on animals which had suffered excision of their temporal lobes led to the introduction of temporal lobectomy for the treatment of some forms of psychomotor epilepsy and to the recognition that activity in structures within the temporal lobe is an important determinant of the emotional state. Impulses from the temporal lobe pass to the hypothalamus (where they influence, and are influenced by, the autonomic system in the manner just described) and thence to the rest of the cortex where they 'set' the emotional tone and colour the conscious response to incoming information. The parts of the temporal lobe (particularly the amygdaloid nuclei) and the cortical areas on the medial surface of the hemispheres whose activity is involved in the control of autonomic and emotional responses are collectively known as the limbic system. The word *limbic* means border and the limbic cortex encircles the diencephalon—the thalamus and hypothalamus. It was previously and less appropriately known as the rhinencephalon or 'nose-brain'.

The hypothalamus contains particularly large amounts (relative to the rest of the brain) of nearly all the neurohumoral substances which have so far been identified. Although these substances are probably not all directly concerned in the regulation of hypothalamic function, their presence in this region of the brain suggests that central nervous activity in which the hypothalamus participates is likely to be particularly susceptible to the action of drugs. Thus, the parts of the brain most directly concerned in the regulation of consciousness, emotional states and the extrapyramidal system are drug sensitive. The existence of a large number of compounds with a psychotropic action and the frequent occurrence, with these compounds, of autonomic and extrapyramidal side effects is thus, in a general way, understandable though, as will be seen, it is difficult to explain the action of a specific drug or group of drugs in terms of its intervention in a particular

biochemical system or in the transmission processes of a particular set of neurones.

Another neurophysiological concept which merits some attention is that of the blood-brain barrier. It is well known that some blood-borne substances do not pass into the brain and while there is little doubt that this is due to the operation of metabolic barriers, some caution should be exercised when assessing the results of experiments in which the blood-brain barrier might have been operative. The barrier is probably not equally impenetrable in all parts of the central nervous system (there is evidence that it is relatively permeable in the hypothalamic region[7] and in the area postrema[8]) and its effectiveness may vary with the experimental conditions, and with the species of animal used. In particular, when two substances are administered together, the presence of one may alter the ability of the other to penetrate into the brain. A particularly challenging review of the physiological aspects of the blood-brain barrier is that of Dobbing[10].

HUMORAL FACTORS IN THE BRAIN

Any pharmacologically-active compound which occurs in extracts of nervous tissue can be thought of as a neurohumoral substance. A transmitter substance is one which is directly responsible for the transmission of nerve impulses across synapses or from nerve to muscle, but the assumption which is sometimes made that a neurohumoral substance necessarily has a transmitter function is quite unjustified. This section of the review summarizes the evidence relating to the central transmitter function of the more important neurohumoral factors found in the brain. Some of the more widely quoted experiments have involved the study of the effects of drugs, alone or in combination, on behavioural activity and on individual neurohumoral substances. Attempts to deduce a causative link between changes in the chemical factors and the observed modifications of behaviour are fraught with difficulties since drugs rarely affect only one chemical system and often have actions on mechanisms not directly related to transmission processes. For this reason, more attention is paid in the present discussion to experiments in which the central nervous actions of the putative transmitter substances themselves are studied. This type of investigation provides more clear-cut and less equivocal information concerning the role of the neurohumoral factors. Drug action is more particularly considered in later sections of the review.

The Recognition of Transmitter Substances

It has become customary for reviewers—including the present author—to preface their discussions of neurohumoral processes with a list of the criteria which must be satisfied before a compound can be classified as a transmitter substance. The simple enumeration of these criteria at first served a useful cautionary purpose but recently a tendency has become apparent for experimental results to be interpreted, often uncritically, in such a way as to suggest that the several criteria are being met. In some instances, this has led to the results being accorded a greater significance than they warrant. The present rather widespread use of the terms 'serotoninergic', 'noradrenergic' and 'dopaminergic', for instance, with the implication that the central

function of the monoamines is as unequivocally established as is that of acetylcholine is particularly unfortunate. The usage is not justified by the evidence and it can only mislead the non-specialist reader. The application of the criteria requires a critical appraisal and it is hoped that the discussion in the succeeding paragraphs enables the reader to make this appraisal for himself.

A transmitter substance is present in, and in many instances it is synthesized by, nerves; it is released from the presynaptic terminals of the nerve fibres when they are stimulated. On release, it produces changes leading to the excitation or inhibition of neurones standing in synaptic relationship with those from which it has been liberated and after exerting its physiological action, it is inactivated by enzymatic destruction, by recombination into an inactive form in the presynaptic terminals or by diffusion from the region of the synapse.

The changes produced in the subsynaptic membrane by transmitter substances are now well characterized. Excitatory transmission involves a local depolarization of the affected neurone and an increase in the permeability of its membrane to all ions. Inhibition provides a more complex situation, for two types of inhibitory process have been described. In postsynaptic inhibition, the inhibitory transmitter causes an increased permeability of the neuronal membrane to potassium or chloride ions. This results in an increase in the resting potential of the cell (hyperpolarization) which thereby becomes stabilized and unable to fire impulses. In presynaptic inhibition, impulses in the inhibitory fibres interrupt the arrival of impulses in the fine terminals of excitatory fibres. Thus, in one form of inhibition there is a reduction in the excitatory bombardment of the neurone; in the other type, the excitability of the neurone itself it depressed. Much of our knowledge concerning the membrane changes underlying excitation and inhibition comes from the work of Eccles and his colleagues, who have also provided a useful pharmacological test for differentiating between the two types of inhibition: postsynaptic inhibition is blocked by strychnine, presynaptic inhibition is blocked by picrotoxin. Eccles' extensive work is summarized in his most recent book[11].

The criteria now to be considered are based on these concepts of the processes of synaptic transmission.

Transmitter substances must be present in nerve fibres, particularly the presynaptic terminals and must be released therefrom on stimulation. The presence of a substance in localized areas of the central nervous system is usually taken as presumptive evidence that the substance has a humoral function particularly in relation to the physiological activity of the regions in which it is found. However, it must be remembered that the one substance—acetylcholine—for which a transmitter function has been clearly established, is quite widely distributed in the nervous system. It is not present in quantity in all nerve fibres—this circumstance, indeed, forms one of the most telling arguments for the existence of non-cholinergic transmission—but its distribution is not obviously related to any one functional system. Thus, cholinergic transmission is the rule in both sympathetic and parasympathetic ganglia and there is good evidence for the presence of cholinergic neurones in the reticular and extrapyramidal nuclei, in the principal motor and sensory tracts, in the

hypothalamus and in several cortical and subcortical regions. While it is reasonable to expect that the transmitter substance liberated at the post-ganglionic terminals of sympathetic nerves should differ from that liberated by parasympathetic nerves, mirroring the different peripheral effects of sympathetic and parasympathetic stimulation, no such consideration can apply to central transmission. Central nervous activity consists exclusively of the transmission of nerve impulses along nerve fibres and across synapses and there is no reason why the details of this activity should vary with the functional system being served. The fact that the central nervous system employs more than one transmitter substance is probably determined by the complexity of synaptic organization rather than by the existence of different functional systems.

A study of the distribution, in the mammalian central nervous system, of the various substances discussed in this review reveals that none—except γ-aminobutyric acid which has general metabolic as well as humoral functions—is as widespread as acetylcholine and there is no general tendency for fibres deficient in acetylcholine to contain correspondingly large amounts of any of the other substances. This is an important point, since the non-cholinergic excitatory transmitter should be found in at least a number of acetylcholine-free nerve tracts. Instead, it appears that some areas of the nervous system contain a multiplicity of humoral substances while others have none at all.

The distribution of the various humoral factors discussed is not considered in detail here since the information is available elsewhere. The interested reader, wishing to confirm the validity of the assertions just made, is referred particularly to the recent book by Garattini and Valzelli[12] in which all the available data are collected together in the most comprehensive tables yet published.

It must also be remembered that in the brain, non-neuronal elements (the glial tissue) outnumber neurones at least tenfold and that some of the substances found 'in the nervous system' might well occur in glial cells. The intrinsic activity of the glial cells, the local control of blood vessels and the production of local hormones for the hypothalamo-hypophyseal system might all involve the participation of humoral factors, to which class some of the substances currently classed as transmitters might belong.

The demonstration that a substance is released on stimulation of a tract of nerve fibres provides useful evidence that it is present in nervous rather than in glial tissue. Its transmitter function becomes more likely if it can be shown that the amount released is sufficient to stimulate or inhibit neighbouring nerve cells. Recent advances in histochemistry have permitted the more accurate localization of neurohumoral substances not only between neurones and glial tissue, but also within the subcellular components of individual cells.

Application of the presumed transmitter substance to a neurone must produce the characteristic change in membrane permeability. The *sine qua non* of transmitter action is the production of the membrane changes characteristic of excitation or inhibition. The multibarrelled microelectrode allows active substances to be applied, by iontophoresis or microinjection, directly to the neurone while the membrane potential is simultaneously recorded. It was hoped that the

application of this technique would lead to quick identification of new transmitter substances. This hope has not been entirely realized, for it has become clear that neurones can show excitatory or inhibitory responses to a wide variety of compounds, many of which are demonstrably not transmitter substances at the neurones under test. The lack of a response to iontophoretic application might be equally misleading, for Eccles has drawn attention to the possible existence of synaptic barriers which could prevent the access even of locally applied material[11].

The pharmacological actions of the putative transmitter and of substances which interact with it must be consistent with its presumed transmitter function. The pharmacological effects of acetylcholine are entirely consistent with its physiological functions—thus it excites the cerebral cortex and inhibits spinal reflexes. If production or liberation of acetylcholine is interfered with, transmission is inhibited. Impairment of function parallels acetylcholine depletion. Equally predictable events follow the administration of drugs such as blocking agents anticholinesterases. As will become evident, such clear-cut relationships are rarely found with the other substances discussed in this review and the subsidiary hypotheses invoked to explain the action of substances related to proposed transmitter substances should be closely examined.

Modulators of Transmission

It is clear that only a few of the many pharmacologically active compounds found in the central nervous system will prove to be transmitter substances in the strict sense of that term. A problem, to which insufficient attention has been paid, concerns the function of those neurohumoral substances to which no transmitter action can be ascribed. It is usually suggested that they are modulators of transmission or 'neuromodulators' which operate by 'modifying function and output (of transmitters) by changing the environment or the metabolism of the neurones either directly or through effects on the surrounding neuroglia' (Everett[13]). Although it is easy to propose ways in which neuromodulator activity might be effected, experimental evidence for the existence of modulator activity, particularly in the central nervous system, is almost completely lacking. Since the properties to be expected of modulator substances are so ill-defined and since modulator action could be effected by a variety of mechanisms, it is impossible to provide criteria by which a neuromodulator might be recognized. The reader is advised to keep an open mind on the subject while remembering that many investigators, armed with optimism rather than experimental evidence, tend to assign the role of modulation to compounds that have not fulfilled the hopes which led to their being originally cast as transmitter substances.

Such evidence as is available concerning the possible modulator action of individual neurohumours is discussed in the appropriate sections of this review.

Acetylcholine

Acetylcholine occupies a unique position among neurohumoral substances for it is the only one which has been conclusively shown to have a transmitter function at central synapses. The relevant evidence has been extensively

summarized on a number of occasions[11,14–17]: because of its general acceptance, it needs only cursory treatment here.

Although the transmitter function of acetylcholine has been most intensively studied at peripheral sites; notably at the junction between motor nerves and striated muscle, a detailed analysis has been made of its action at one type of central synapse. The circumstantial evidence that it has a similar function elsewhere in the central nervous system is very strong.

Throughout the length of the spinal cord, small neurones (the Renshaw cells) located in the region of the anterior horns, are functionally connected with the anterior horn cells (the motoneurones). The Renshaw cells are inhibitory to the motoneurones so that when stimulated they have the effect of reducing the discharge of nerve impulses along the motor nerves. The physiological stimulus to the Renshaw cells in its turn comes from the motor nerves. Branches leave the motor nerve fibres within the cord and make synaptic contact with the Renshaw cells. The system as a whole presumably operates as a self-regulating mechanism, intense activity in the motor nerves tending to reduce the neuronal stimulation to which this activity is due. Since motor nerves liberate acetylcholine at their peripheral terminals, it seemed reasonable to assume that their collateral fibres would do the same and that synaptic transmission between these collaterals and the Renshaw cells would be cholinergic in nature. Micro-electrode studies have confirmed that this is so[18,19]. Acetylcholine depolarizes the Renshaw cells; the depolarization brought about as a result of physiological activation of the Renshaw cells is blocked by the acetylcholine-blocking agents, curare and dihydro-β-erythroidine. Anticholinesterases inhibit the destruction of acetylcholine and prolong the action of acetylcholine and physiological stimulation on the Renshaw cells. Renshaw cells are stimulated by other choline esters, by tetramethylammonium and by nicotine, all compounds which mimic acetylcholine action elsewhere. At both the neuromuscular junction and at the Renshaw cell synapses, the amount of acetylcholine required to stimulate the cells is no greater than that liberated by physiological activity in the motor nerves. In the intact animal, acetylcholine, administered by intra-arterial injection, inhibits spinal reflexes[20]. This pharmacological action is consistent with the results of the micro-electrode studies on single cells. The criteria for transmitter action are thus completely met.

Spinal neurones other than Renshaw cells are not depolarized by acetylcholine[21]. This observation not only underlines the physiological significance of the Renshaw cell sensitivity but it also adds support to the now generally held view that acetylcholine is not the transmitter substance at all synapses in the cord.

Evidence that acetylcholine is a chemical mediator of synaptic transmission elsewhere in the central nervous system is circumstantial but convincing. Acetylcholine is the transmitter substance from motor nerves, preganglionic autonomic fibres and the postganglionic fibres of the parasympathetic system to their respective effector cells. In all these fibres, the activity of choline acetyltransferase (choline acetylase), the enzyme responsible for the synthesis of acetylcholine, is high. Conversely, its activity is negligibly low in sensory nerve fibres[22] and transmission from these fibres is demonstrably non-cholinergic in nature[23]. It is reasonable, therefore, to

assume that the distribution of choline acetyltransferase in the central nervous system reflects the distribution of cholinergic fibres. This distribution was first studied by Feldberg and Vogt[24] who pointed out that there was a tendency for presumed cholinergic fibres to alternate with non-cholinergic fibres. Although there are now known to be many exceptions to this generalization, it does serve the useful purpose of emphasizing the wide distribution of cholinergic neurones. These neurones are not restricted to particular physiological systems. So wide a distribution has not been found for any other humoral substance. It should also be noted that the choline acetyltransferase and acetylcholine contents are high in many of those parts of the brain (particularly the caudate nucleus, the thalamus and the hypothalamus) in which noradrenaline, dopamine and 5-hydroxytryptamine are localized[12]. The sensitivity to acetylcholine of the cells of the supraoptic nucleus of the hypothalamus[25] and the presence of acetylcholine in fibres in synaptic contact with the nucleus[24] certainly suggest that transmission is cholinergic in the area of the hypothalamus at least. Acetylcholine is thus as likely as the monoamines to be involved in the basic mechanism of action of psychotropic drugs. The recent popularity of studies on the monoamines has tended to obscure this fact.

Iontophoretic application has shown that cells sensitive to acetylcholine are widespread throughout the brain. They are found in the medulla[26], the mid-brain[27,28] (including the reticular system), the thalamus[29], the lateral geniculate body[30], the cerebellum[31] and the cerebral cortex[32–34]. In these areas of the central nervous system, up to 30 per cent of the cells seem to be excited by acetylcholine, though physiological activation of all these neurones is not necessarily exclusively effected by acetylcholine. The major part of the nervous inflow to the lateral geniculate body, for instance, is carried by the optic nerves, which contain virtually no acetylcholine[22]. Synapses on the lateral geniculate body must consequently be predominantly non-cholinergic and the sensitivity of some of its neurones to acetylcholine may be of purely pharmacological interest. It may, on the other hand, reflect a physiological event for there is evidence for the existence of acetylcholine-rich fibres connecting the two geniculate bodies[24].

The apparently wide distribution of cholinergic neurones presumably accounts for the ease with which it is possible to demonstrate drug-induced changes in the acetylcholine content of brain and in the rate of its release from the irrigated cerebral cortex.

As is well known, acetylcholine is destroyed by the cholinesterases, of which there are two types (strictly, two groups), 'true', 'specific' or acetylcholinesterase and 'pseudo', 'non-specific' or butyrocholinesterase. Acetylcholinesterase preferentially hydrolyses acetylcholine and other acetyl esters while butyrocholinesterase is more active against the higher homologues of acetylcholine, particularly propionylcholine and butyrylcholine. In striated muscle, autonomic ganglia and in the central nervous system, the predominant form is true cholinesterase but its localization differs in these areas. At the neuromuscular junction it is found chiefly on the end-plate (that is, postsynaptically)[35] where is it ideally placed for hydrolysing acetylcholine which has served its immediate transmitter function. In antonomic ganglia, cholinesterase is located in the preganglionic fibres[36] (that is, presynaptically)

where it seems to be involved in the mechanism of acetylcholine release. Inactivation of acetylcholine after its reaction with the postsynaptic receptor site appears likely to occur as a result of simple diffusion from the ganglion[37]. In the central nervous system, cholinesterase is found at both presynaptic and postsynaptic sites.

The spleen in some species contains relatively large amounts of propionylcholine[38]. This fact, together with the presence of butyrocholinesterase in the central nervous system and the pharmacological activity of the choline esters, suggests the possibility that substances related to acetylcholine may also participate in the processes of synaptic transmission. Although choline esters other than acetylcholine, as well as other substances with acetylcholine-like activity, have been reported to occur in the brain[39–41], pharmacological and biochemical analyses of brain extracts have failed to confirm these claims[42–45]. Similar reports of the existence of acetylcholine-like substances in peripheral nerve extracts[46] have also proved impossible to confirm[47]. The physiological substrate of pseudocholinesterase in the nervous system—if, indeed, it is not acetylcholine—remains unknown. Attempts to solve the problem by differential inhibition of the two cholinesterases in the brain have so far proved unsuccessful.

The Monoamines

Metabolism of the monoamines

It has been accepted for many years that the catecholamines of nervous tissue and the adrenal medulla arise from phenylalanine by the pathway shown in *Figure 5.1.* Many of the enzymes involved in this reaction sequence

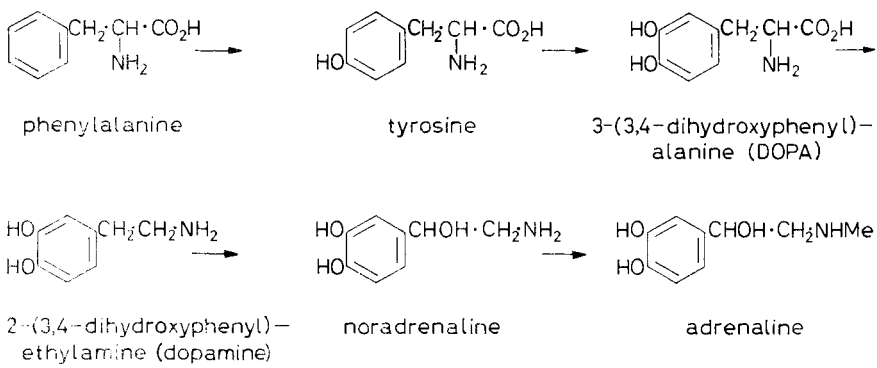


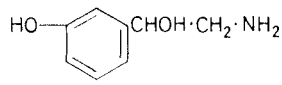
Figure 5.1. The formation of noradrenaline and adrenaline

have been characterized. Phenylalanine hydroxylase appears to consist of two enzymes which have been found together in the liver[48]. It is possible that the next stage proceeds non-enzymatically since no enzyme which can promote the hydroxylation of tyrosine has been identified. The next two steps require the participation of DOPA decarboxylase and dopamine-β-oxidase, respectively. Both enzymes are present in the adrenal medulla and in nervous tissue[49–52]. Phenylethanolamine *N*-methyltransferase transfers

the methyl group of *S*-adenosyl methionine to the amino group of noradrenaline[53].

The physiological significance of the scheme outlined is emphasized by the observation that phenylalanine, tyrosine, DOPA and dopamine were all converted to adrenaline when they were incubated with homogenates of adrenal medulla[54]. A particularly interesting finding is that of Goodall and Kirschner[50] that, whereas labelled DOPA became transformed into noradrenaline when it was incubated with extracts of sympathetic nerves, no such conversion occurred when homogenates of the vagus nerves were used.

The enzymes involved in the formation of the catecholamines are of low specificity. DOPA decarboxylase, or an enzyme closely akin to it, is concerned in the formation of 5-hydroxytryptamine[55,56]; dopamine-β-oxidase has been shown to be capable of hydroxylating the β-carbon atom of a number of tyramine derivatives[57,58] and phenylethanolamine *N*-methyltransferase is equally unselective in its *N*-methylation of noradrenaline derivatives[59]. This lack of specificity suggests the possibility that alternative pathways for the formation of noradrenaline and adrenaline might exist *in vivo*. Some of the putative intermediaries in these other pathways have been shown to occur naturally and one of them, octopamine (*I*) is found in the brain[60].



(I) octopamine

Adrenaline is the major component of the hormonal secretion of the adrenal medulla and in the adrenal gland noradrenaline can be regarded simply as a precursor of the active substance. In sympathetic nerves, however, catecholamine synthesis proceeds no further than noradrenaline, which exists there as a transmitter substance in its own right, with properties different from those of adrenaline. Noradrenaline and small amounts of adrenaline are present in the brain also, but in addition, certain areas of the brain contain amounts of dopamine quite out of proportion to their noradrenaline content and there is suggestive evidence that dopamine has an independent neurohumoral function. The unique situation thus arises that one, or probably two of the precursors of adrenaline have specialized functions of their own.

The other physiologically important monoamine is 5-hydroxytryptamine (serotonin or 5-HT). It is formed from tryptophan via 5-hydroxytryptophan (5-HTP) (*Figure 5.2*). The nature and properties of tryptophan-5-hydroxylase is still obscure, though the hydroxylation of tryptophan *in vivo* has been demonstrated[61]. There is no clear evidence that this conversion occurs in brain tissue. The decarboxylation of 5-HTP, however, takes place in brain and the decarboxylating enzyme is found in all cerebral areas which contain 5-hydroxytryptamine. 5-HTP decarboxylase is closely related to, if not identical with, DOPA decarboxylase[55,56] and agents which inhibit dopamine formation similarly inhibit the production of 5-hydroxytryptamine. There

has been a suggestion that the formation of 5-hydroxytryptamine proceeds by way of tryptamine rather than 5-HTP, but this now seems unlikely[62].

Metabolic degradation of the catecholamines occurs either through deamination as a result of monoamine oxidase activity or through *O*-methylation by the enzyme catechol-*O*-methyltransferase. The major metabolites produced by these enzymes are shown in *Figure 5.3.* In addition, small amounts of catechol amines are excreted unchanged[63] or as conjugates with

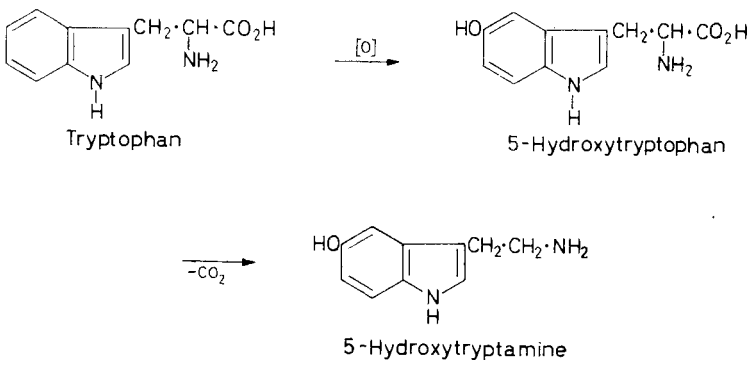


Figure 5.2. The formation of 5-hydroxytryptamine

glucuronic acid[64]. Adrenaline can also be *N*-methylated by phenylethanolamine-*N*-methyltransferase and the *N*-methyladrenaline thus formed can, like adrenaline itself, be *O*-methylated by catechol-*O*-methyltransferase. It has also been suggested that adrenaline may be converted into adrenochrome and adrenolutin (*Table 5.6*). These compounds, which have psychotomimetic properties, are considered later; here it need only be said that the evidence that they are produced in man is unconvincing[65,66]. Much of our recent knowledge concerning the metabolism of the catecholamines comes from the work of Axelrod and his colleagues. His reviews survey the subject in more detail than is possible here[67,68].

Axelrod has shown that, in man, circulating adrenaline and noradrenaline are metabolized predominantly by catechol *O*-methyltransferase, approximately 70 per cent of an administered dose being inactivated by *O*-methylation and 20 per cent by deamination. Generally similar results were obtained in studies on other mammals. The principal urinary metabolite of the catechol amines is 3-methoxy-4-hydroxymandelic acid.

The greater importance of *O*-methylation in the inactivation of circulating catechol amines is due to the fact that, in the liver, where most of the breakdown takes place, the activity of catechol *O*-methyltransferase is very much higher than that of monoamine oxidase. This relationship does not, however, apply to all other organs: in the heart, for instance, monoamine oxidase is about five times as active as the *O*-methyltransferase, and monoamine oxidase also plays a part in catechol amine metabolism in the brain.

Catechol-*O*-methyltransferase is found in cell cytoplasm and monoamine oxidase occurs in the mitochondria. In the mitochondria in the brain, noradrenaline also occurs in a bound form, probably in two 'compartments',

in one of which noradrenaline is more tightly bound than in the other. The loosely bound noradrenaline in sympathetic nerve fibres is the immediate source of that liberated by nerve impulses. The liberated noradrenaline is

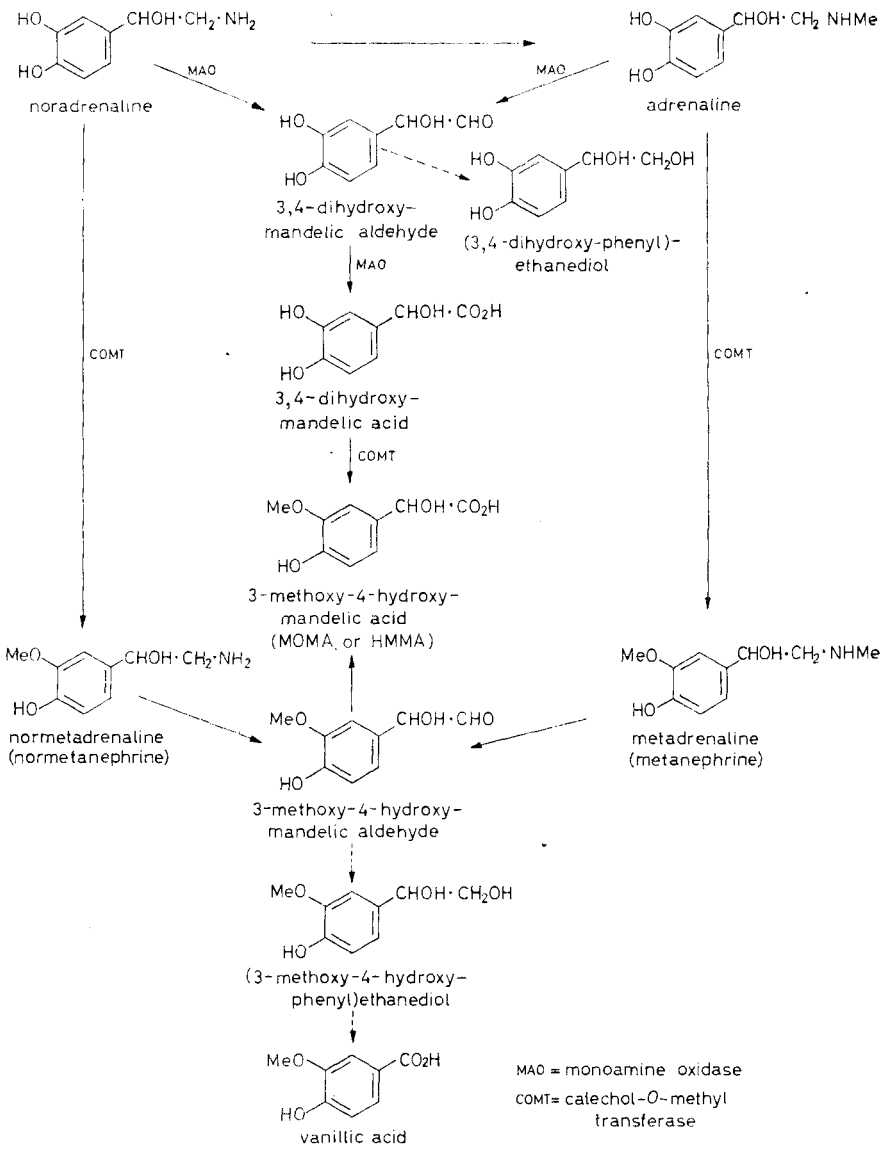
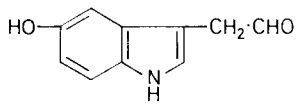


Figure 5.3. The breakdown of the catecholamines[67,68]

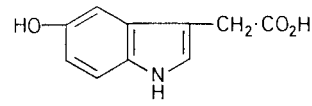
either destroyed by catechol *O*-methyltransferase or is reincorporated in the neurones from which it was released. Intracellular noradrenaline in the 'unbound' form is destroyed by monoamine oxidase.

Dopamine is also inactivated by monoamine oxidase to give 3,4-dihydroxyphenylacetic acid which is further metabolized by catechol *O*-methyltransferase to give 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid)[69].

A number of routes by which 5-hydroxytryptamine may be broken down or otherwise inactivated have been established[70]. The most important involves monoamine oxidase, which converts 5-hydroxytryptamine into 5-hydroxyindoleacetaldehyde (*II*). Catabolism is completed by an aldehyde dehydrogenase which forms 5-hydroxyindoleacetic acid (*III*), the principal urinary metabolite of 5-hydroxytryptamine. Like noradrenaline, 5-hydroxytryptamine can also be conjugated with glucuronic and sulphuric acids.



(II) 5-hydroxyindoleacetaldehyde



(III) 5-hydroxyindoleacetic acid (5-HIA)

Noradrenaline

In the peripheral nervous system, noradrenaline is accepted as the alternative transmitter substance to acetylcholine, mediating the transmission of impulses from most of the postganglionic sympathetic fibres to their effector organs. The evidence that noradrenaline also has a transmitter function in the central nervous system is less clear.

Noradrenaline (with very much smaller amounts of adrenaline) has a patchy distribution in the central nervous system[12]. In nerve cells containing noradrenaline the amine, like acetylcholine, is found in the presynaptic vesicles[71–73]. The largest amounts (about 1 μg/g of tissue) are found in the hypothalamus and in the area postrema of the fourth ventricle; the grey matter of the medulla and mid-brain contains about one third of this quantity. Small amounts of noradrenaline are widely distributed in other parts of the nervous system but it seems reasonable to assume that its neurohumoral function will prove to be particularly associated with central nervous actions which are mediated through hypothalamic and brain stem centres. In this connection, it should be pointed out that all areas of the hypothalamus contain noradrenaline. The anterior nuclei (which control parasympathetic functions) contain larger quantities than the posterior nuclei from which the sympathetic outflow originates. It is sometimes rather too readily assumed that, because noradrenaline is responsible for bringing about the effects of sympathetic stimulation at the periphery, it will be necessarily and exclusively concerned with the activity of those hypothalamic neurones which control the sympathetic outflow. If brain content is any indication of the importance of a neurohumoral substance, noradrenaline might be expected to be equally important in the regulation of parasympathetic activity.

Any assessment of the central effects of noradrenaline following its parenteral administration has to be made with care since the blood-brain barrier has a limited permeability to the catecholamines. The feelings of anxiety and apprehension reported by subjects who have received adrenaline

are not noticed after noradrenaline, though the latter compound is more important in the brain. The peripheral autonomic effects of adrenaline, in those parts of the body from which sensory impulses are likely to become charged with emotional affect, are more pronounced than those of noradrenaline, and it seems that many of the 'central' effects of adrenaline administration may be due to stimuli of peripheral origin. Nevertheless, the blood-brain barrier in the hypothalamic region seems to be more permeable to the catecholamines than it is elsewhere in the brain[7] and the results of experiments in which blood pressure changes were suitably compensated suggest that both adrenaline and noradrenaline have an 'alerting' action when administered intra-arterially or intravenously, particularly in the sleeping animal[74–76]. Since noradrenaline occurs in those parts of the brain containing a large number of reticular synapses, the alerting effect provides presumptive evidence that transmission in the reticular system may be mediated or influenced by noradrenaline. It must, however, be added that it has not been possible to demonstrate the liberation of noradrenaline from the brain during stimulation of the ascending reticular system[77].

Although the catecholamines are usually thought of as being excitatory in nature, they often have depressant actions. Even on the reticular system, 'deactivation' as well as activation by noradrenaline has been observed in the course of a single experiment[78] and a study of the reported actions of the catecholamines on the electrical activity of the brain reveals a bewildering mixture of stimulated, depressed and unchanged electroencephalograms. Some of these conflicting results are summarized by Longo[79]. In some situations the catecholamines are invariably depressant. Rothballer[76] has pointed out that the first report concerning the central actions of intra-arterially injected adrenaline considered the possibility that it might be used as an anaesthetic and the injection of small amounts of adrenaline or noradrenaline into the cerebral ventricles causes a condition resembling light anaesthesia[80]. Marrazzi has studied one aspect of the inhibitory actions of the monoamines in some detail. He stimulates the visual cortex and records the potentials evoked *via* the corpus callosum in the corresponding area of the cortex of the contralateral side. Acetylcholine facilitates these potentials but adrenaline and noradrenaline inhibit them[81,82]. 5-Hydroxytryptamine, lysergic acid diethylamide, amphetamine, adrenochrome and other compounds have a similar inhibitory action. On the basis of these experiments, Marrazzi believes that the monoamines have essentially inhibitory functions in the brain, but in view of the low noradrenaline and negligible 5-hydroxytryptamine content of the visual cortex, the physiological significance of these observations is not clear.

The application of noradrenaline, by iontophoretic injection, to single central neurones has produced information which partially clarifies an otherwise confusing situation. In this type of experiment, neurones are directly studied and the nature of any change produced can be described in terms of membrane changes which can be compared with those known to be associated with transmitter action. The possibility that the observed effects are being complicated by concurrent peripheral stimulation or inhibition is excluded. Several groups of workers have shown that in a number of areas of the brain and in the spinal cord, noradrenaline has a predominantly

depressant action which is of postsynaptic origin[34,83–86]. It is interesting, in connection with its action on the spinal cord, that some noradrenaline has been found in the descending tracts[87] of the spinal cord and that stimulation of the isolated mouse cord causes the liberation of noradrenaline into the bathing medium. Single neurones of the reticular system on the other hand are usually excited by noradrenaline, though inhibition is sometimes seen[27,28]. The excitatory and inhibitory actions of noradrenaline seen in the other experiments referred to, are thus adequately explained. There is at present no evidence that the action of noradrenaline on the postsynaptic membrane reflects the physiological situation. It has already been emphasized that the neuronal membrane responds non-specifically to a large number of substances and it may be significant, that in most of the areas so far investigated, none of the known blocking agents inhibit the action of iontophoretically-applied noradrenaline[34]. This suggests either that the cells do not carry specific noradrenaline receptors or if they do, that their nature is very different from that at peripheral sites. The former possibility seems to be the more likely, for some at least of the substances that block the peripheral actions of other neurohumours (acetylcholine and 5-hydroxytryptamine, for instance) are effective centrally. Moreover, the response to noradrenaline in the mitral cells of the olfactory bulb is partially inhibited by noradrenaline-blocking agents[84] and there is evidence, from other types of experiment, for the existence in neurones of the reticular formation of receptors similar to, but not identical with, peripheral adrenaline receptors[75].

The possibility cannot be excluded that the actions of noradrenaline, as revealed by iontophoretic studies, reflect its physiological function in some areas but not in others. The large amounts of noradrenaline in the hypothalamus and mid-brain suggest that the observed stimulation of reticular neurones by noradrenaline points to its having an excitatory function in the brain stem. If it has a transmitter function elsewhere, it may well be inhibitory in nature. It is clear that even the evidence that noradrenaline is a transmitter in the reticular system is not nearly so convincing as that which supports the argument that acetylcholine is a mediator of transmission at central synapses.

There is some evidence which has been reviewed elsewhere[89] that, at the neuromuscular junction and at synapses in autonomic ganglia, adrenaline and noradrenaline influence transmission without initiating the transmission process. At these sites, the catecholamines may act as modulators of transmission and the possibility that they may have a similar function in the central nervous system must be borne in mind. Direct evidence for such a central function is, however, lacking.

5-Hydroxytryptamine

As long ago as the middle of the nineteenth century, physiologists were aware that blood serum contained a vasoconstrictor substance, but this material ('vasotonin' or 'serotonin') was not isolated in a pure form until 1948[90]. Rapport shortly afterwards identified serotonin as 5-hydroxytryptamine[91]. A few years later Erspamer demonstrated that 'enteramine', an active material he had detected in extracts of intestinal mucosa almost 20 years earlier, was also 5-hydroxytryptamine[92]. Subsequently, 5-hydroxytryptamine was found to be widely distributed in tissues and to possess a

variety of pharmacological actions. These findings provided the stimulus for a large amount of experimental work, the volume of which was enormously increased following the suggestion[2,3] that the hallucinogenic activity of lysergic acid diethylamide (*Table 5.6*) might be due to it antagonizing 5-hydroxytryptamine in the brain. Much recent work has attempted to establish a central transmitter function for 5-hydroxytryptamine, though only a minor part of the body's store is found in the central nervous system. Extensive reviews of the recent literature are available; some[93–96] covering all aspects of the subject while others[97,98] are exclusively concerned with the role of 5-hydroxytryptamine in the nervous system.

A large proportion of the experimental work has involved the analysis of the effects of drugs which are thought to interfere with the synthesis, storage, release or breakdown of 5-hydroxytryptamine. This type of evidence is discussed later (p. 296). Physiological studies, summarized here, have yielded few results to justify the enthusiasm provoked by some of the pharmacological investigations.

The distribution of 5-hydroxytryptamine in the central nervous system has been studied among mammals, in the rat, cat, dog, monkey, rabbit, ox and man. In all these species, it is present in largest amounts in the thalamus, the hypothalamus and the mid-brain[12]. The amounts found in these areas have varied, with the investigator and with the species studied, between 0·4 and 2·5 μg/g. This distribution recalls that of noradrenaline, though there is some evidence from the rat, that the fibres containing 5-hydroxytryptamine are distinct from those containing noradrenaline[99]. Moreover, 5-hydroxytryptamine is found in the thalamus, the basal ganglia and the hippocampus in much larger amounts relative to the hypothalamic content, than is noradrenaline. The 5-hydroxytryptamine of nerve cells seems to be concentrated in the presynaptic terminals[100–102], and not necessarily in the presynaptic vesicles[103].

On iontophoretic application to single cortical neurones, 5-hydroxytryptamine has an inhibitory action similar to that found with the other monoamines[34,83,104]. It also inhibits transcallosally-evoked potentials in the optic cortex[81,82]. It has a more powerful inhibitory action than has either noradrenaline or dopamine on synaptic transmission in the lateral geniculate body[30]. Earlier reports had suggested that it had no action on spinal neurones[105], but a recent study suggests that 5-hydroxytryptamine, like noradrenaline, exerts some of the electrogenic actions to be expected of the inhibitory transmitter substance[86]. These results indicate, that so far as its action on individual neurones is concerned, 5-hydroxytryptamine has inhibitory properties. The physiological significance of the observations is less clear since there is no evidence that 5-hydroxytryptamine is liberated (except in the spinal cord[106]) in the course of normal neuronal activity in the neighbourhood of any of the cells on which it has been shown to be active.

In most animals, 5-hydroxytryptamine appears to cross the blood-brain barrier with difficulty (the mouse provides an exception[106]) but the behavioural effects of 5-hydroxytryptophan (which is decarboxylated in the brain to 5-hydroxytryptamine) have been extensively studied in a number of species. Although the various reports show some differences in detail, it is

generally true to say that small doses of 5-HTP cause sedation[107] while larger doses lead to excitation[106,108,109]. The behavioural effects are accentuated and the rise in brain 5-hydroxytryptamine content is increased if a monoamine oxidase inhibitor is administered with the 5-HTP. Intraventricular injection of 5-hydroxytryptamine produces some of the effects of parenteral administration of 5-HTP, suggesting that these are of central origin[80]. It should be added that the 'excitement' produced by 5-HTP is characterized by increased locomotor activity and often takes the form of an 'obstinate aimless progression', the animals failing to avoid or to be affected by obstacles in their path[98]. This contrasts with the aggressiveness shown by animals that have received DOPA; suggesting that dopamine and 5-hydroxytryptamine may have different loci of action.

The administration of a monoamine oxidase inhibitor alone also produces an increase in the 5-hydroxytryptamine content of the brain but this is associated with much smaller behavioural changes than are seen when 5-HTP is also given. The monoamine formed from exogenous 5-HTP is probably only slowly taken up by the storage granules and it can therefore exert a prolonged pharmacological effect before incorporation. Endogenously formed 5-hydroxytryptamine, on the other hand, is produced within the nerves and might not be released in the course of normal neuronal activity. If this is the explanation of any discrepancy between the behavioural responses in animals whose brain 5-hydroxytryptamine content has been elevated by different methods, it might imply that the normal site of action of the amine is intraneuronal rather than synaptic. The liberation of 5-hydroxytryptamine in the brain has never been demonstrated and it may perhaps be involved in the regulation of ionic movements inside the cell[98], although if this be the case, it will become necessary to explain why only cells in restricted areas of the brain require the assistance of the 5-hydroxytryptamine mechanism to regulate an activity common to all neurones.

The amount of 5-hydroxytryptamine found in the central nervous system is determined to some extent by dietary factors. Boullin[111] fed rats on a tryptophan-deficient diet and produced animals whose brain 5-hydroxytryptamine was no more than 20 per cent of that in pair-fed control animals. Some of the deficient animals exhibited behavioural changes including tremor, ataxia and convulsions. These changes were not reversed by 5-HTP, a result which does not conflict with the view that the physiological site of action of 5-hydroxytryptamine is intraneuronal.

The actions of 5-hydroxytryptamine and of 5-HTP on the spontaneous electrical activity of the cortex are usually inhibitory (flattening of the record) with some tendency to excitation at higher dose levels. Spike activity (indicating a discharging area) is seen in some subcortical structures[112–114].

The many investigations which have examined *inter alia*, the effect on 5-hydroxytryptamine metabolism of convulsant and anticonvulsant drugs, of electroshock and insulin coma, have yielded results which though interesting throw little light on the physiological function of 5-hydroxytryptamine[115].

Feldberg and Myers[116] found that the intraventricular injection of 5-hydroxytryptamine increased body temperature while adrenaline and

noradrenaline depressed it. They suggest that the regulation of body temperature may depend on the co-ordinated release of the monoamines from hypothalamic structures. The experiments of Feldberg and Myers were carried out on conscious cats. Other workers on the other hand, have reported that in conscious rabbits, intraventricular 5-hydroxytryptamine depresses body temperature while noradrenaline elevates it[117].

The interesting observations by Woolley[118] that 5-hydroxytryptamine regulates the rate and extent of oligodendrocyte contractions in culture does not seem to have been developed further. In view of the increasing recognition of the importance of glial cells in cerebral function, the possibility that 5-hydroxytryptamine may have an important extraneuronal function has to be borne in mind. In the present state of our knowledge, however, we have to confess complete ignorance of its physiological role.

Dopamine

The view that dopamine is more than a precursor of noradrenaline dates from the findings of several groups of workers[119–123] that relatively large amounts of dopamine occur in certain areas of the brain, notably the basal ganglia and some related structures. The distribution of dopamine does not parallel that of noradrenaline in the way that might be expected of a precursor. The interest aroused by these observations was sharpened by the discovery, at about the same time, of an association between dopamine deficiency and Parkinson's disease. The latter topic is discussed later in this review.

Although dopamine is found in several invertebrate species[124], it is absent from the brains of birds and amphibians[125] and it may be significant that extrapyramidal control is less well developed in these species than in mammals. In all mammals (including man) so far studied, the major part of the brain's dopamine appears to be localized in the basal ganglia, the thalamus and the hypothalamus. The overall brain content (0·3–0·6 μg/g) and the maximum local content (5–8 μg/g in the caudate nucleus and putamen) show remarkably little variation among different mammalian species[12]. Not all components of the basal ganglia contain equally large amounts of dopamine. In man, for instance, the amounts per gram range from 5–8 μg in the caudate nucleus and putamen to only 0·3–0·4 μg in the globus pallidus and the substantia nigra[122,123]. These differences may be partly explained by the fact that dopamine, like noradrenaline and 5-hydroxytryptamine, reaches its highest concentration in synaptic terminals[73,126] and areas containing the cell bodies of dopamine-containing neurones would be expected to show a lower amine content than regions receiving the axons of these neurones. It is interesting, in this connection, that Carlsson and his colleagues have observed that, in rats, a tract of dopamine-containing fibres passes from the substantia nigra to the corpus striatum and that lesions of the substantia nigra lead to a loss of dopamine from the corpus striatum[127]. Dopamine-containing neurones have also been detected in the retina of the rabbit[128].

The basal ganglia also contain relatively large amounts of 5-hydroxytryptamine, substance *P* and acetylcholine[12]. The caudate nucleus has a higher acetylcholine and choline acetyltransferase activity than has any

other area of the brain. There is some evidence, from the work of McLennan[129], that of the fibre tracts connecting the thalamus to the caudate nucleus, the neurones containing dopamine form an anatomically distinct group from those containing acetylcholine. Stimulation of the anterior ventral nucleus of the thalamus caused the liberation of acetylcholine from the caudate nucleus. Stimulation of the centromedian thalamic nucleus resulted in the liberation of dopamine. The output of both acetylcholine and dopamine was reduced when a cortical area (the sigmoid gyrus) was stimulated at the same time as the thalamus.

Although interest in dopamine is centred on the possibility that it may be involved in the regulation of extrapyramidal function, physiological investigations have been carried out, for the most part, on neurones distant from the basal ganglia. When topically applied to the spinal cord of the cat, dopamine inhibited the knee jerk, a monosynaptic reflex[130]. This action was antagonized by 3,4-dichloroisoprenaline (a compound which blocks adrenaline β receptors) but not by dibenzyline which blocks α receptors. It was also antagonized by strychnine, an interesting observation in view of the probability that strychnine owes its convulsant action to its power of opposing inhibitory transmission. Hyperpolarization is the membrane change characteristic of postsynaptic inhibition and dopamine has been shown to cause this change in the brain of the common snail[131]. It also inhibits some crayfish stretch receptor neurones[132,133]. Although these observations give some indirect support to the view that dopamine may be concerned in inhibitory transmission in the cord, it must be added that Curtis was unable to detect any action of dopamine on mammalian spinal neurones[134]. The dopamine content of the spinal cord is also quite low[135,136]. In contrast to its lack of action on cord neurones, dopamine, applied iontophoretically to cortical neurones, has a depressant action, particularly on cells excited by L-glutamate[83]. The neurones of the lateral geniculate body were also weakly depressed[30,137].

The suggestion that dopamine exerts a central inhibitory action is opposed by observation of its behavioural effects. Dopamine itself does not pass the blood-brain barrier but DOPA, its immediate precursor, does pass through and parenteral administration of DOPA leads to an increase in the dopamine content of brain. After DOPA injection, mice become aggressive, exhibiting fear reactions and a Straub tail[138–140]. Larger doses have been reported to cause catalepsy[140]. As would be expected, the effect of DOPA is increased in animals given monoamine oxidase inhibitors[13]. The sedative actions of a number of tranquillizing drugs (including reserpine, deserpidine and chlorpromazine) are reversed by DOPA[13,138,141,142]. These effects are paralleled by the actions of DOPA on the electrical activity of the brain: in rabbits and cats an alerting reaction has been observed[113,143–145]. DOPA has an emetic action[142,146] and the vomiting caused by the bean *Mucuna capitata* is due to its contained DOPA[147].

In attempting to assess the physiological significance of observations of the type summarized in this section, several points must be borne in mind. Following the administration of DOPA in the doses commonly used (100–500 mg/k), the dopamine content of brain rises to a level well above that in the normal animal. Moreover, since DOPA decarboxylase is found in parts

of the brain which normally contain no dopamine, the distribution of dopamine following DOPA injection differs from that in the normal animal and the effects observed may arise from the action of unphysiologically large amounts of dopamine at sites additional to those at which it is normally active. The exogenous DOPA will also lead to increased amounts of cerebral noradrenaline. When its actions on neurophysiological preparations are being evaluated, it must be remembered that dopamine may affect receptors which normally respond to adrenaline or noradrenaline.

The physiological evidence suggests that dopamine may have a neurohumoral function in the central nervous system but this function cannot yet be defined.

γ-Aminobutyric Acid (4-aminobutyric acid, GABA)

Although neurophysiologists had long recognized—certainly since the work of Sherrington in the early years of this century—the importance of inhibition in the regulation of central nervous activity, it was not until 1953 that the subject began to attract the attention of pharmacologists and biochemists. In that year, Florey[148] reported that extracts of mammalian brain contained both excitatory and inhibitory substances which he named Factor *E* and Factor *I* respectively. Factor *I* inhibited the discharge of impulses from the crayfish stretch receptor and this preparation has since been widely used for the quantitative assay of Factor *I* activity in tissue extracts. Crude Factor *I* antagonized the action of acetylcholine on several pharmacological preparations: it inhibited, on local application to the cord, the stretch reflex of the cat and it protected mice against the convulsive action of strychnine[149,150]. This last observation was very interesting in view of the demonstrated antagonism of strychnine and the transmitter substance of postsynaptic inhibition, but it has proved impossible to confirm[151,152]. Factor *I* was shown to possess some excitatory actions, facilitating the flexor reflex and stimulating the hypoglossal nucleus[150].

In 1957 widespread interest was roused by the report of Bazemore, Elliott and Florey[153] that the Factor *I* activity of mammalian brain was due to its contained GABA. Since then a mass of information concerning the pharmacological actions of GABA has been collected; it has been well reviewed on a number of occasions[153–157].

The view that Factor *I* is identical with GABA has not gone unchallenged. McLennan, particularly, has been active in drawing attention to the lack of a complete parallelism between the properties of Factor *I* and GABA and to the presence, in brain, of other compounds possessing properties similar to those of Factor *I*[158]. He suggested at first that some Factor *I* activity was due to γ-guanidinobutyric acid[159] but more recently he has come to the conclusion that dopamine is a more important component. He bases this view partly on the neuropharmacological actions of dopamine, already referred to (p. 271) and partly on the basis of the differential actions of picrotoxin, chlorpromazine and dibenzyline on crayfish stretch receptor inhibition, due to Factor *I*, GABA and dopamine[132]. Others have suggested that γ-amino-β-hydroxybutyric acid[160] and γ-aminobutyryl choline[161–163] might be important inhibitory principles in brain but the evidence on which these views are based has been questioned[164,165].

Whatever the actual composition of Factor *I*, it is undeniable that the brain contains large amounts of GABA and that the properties of this substance are inhibitory in nature. The neuropharmacology of GABA itself is therefore considered in this section.

GABA was first found in brain in 1950[166–168] but until the work of Bazemore, Florey and Elliott, its function was thought to be exclusively biochemical. In vertebrates, it is restricted to the central nervous system[169], an interesting finding in view of the fact that in these species all inhibition arises centrally. It has a wide and relatively even distribution in the brain, ranging from about 250 μg/g in the cortex to about 550 μg/g in the mid-brain. The high concentration and wide distribution of GABA probably reflect its metabolic function but there is no *a priori* reason why small amounts should not, in addition, have a transmitter action.

GABA is formed from glutamic acid by an essentially irreversible action involving glutamic decarboxylase with pyridoxal phosphate (Vitamin B_6) as a cofactor. GABA also undergoes a transamination reaction with α-keto-glutaric acid, resulting in the production of succinic semialdehyde which itself can be enzymatically oxidized to succinic acid. The transaminase also requires Vitamin B_6 as a cofactor but, unlike glutamic decarboxylase, the enzyme is not confined to the brain. α-Ketoglutaric and succinic acid are both intermediates in the tricarboxylic acid cycle and the GABA reactions just described provide an irreversible shunt which permits the continuing metabolism of α-ketoglutaric acid, independently of the α-ketoglutaric oxidase system (*Figure 5.4*). It is clear that, whether or not GABA has a humoral function, it certainly occupies a unique position in the metabolic systems of the brain.

In the crayfish stretch receptor GABA mimics perfectly the actions of the putative inhibitory transmitter substance, causing an increased membrane permeability to potassium and chloride ions. It is not clear, however, whether crayfish nerve contains GABA: early studies failed to demonstrate its presence in crustacean nerves[170], but more recent reports suggest that it does occur in these nerves, particularly in inhibitory fibres[171]. The situation in the vertebrate central nervous system is more difficult to assess: iontophoretic application of GABA produces a non-specific depression of all membrane responses, excitatory and inhibitory alike. Hyperpolarization and the permeability changes characteristic of inhibitory transmitter action have not been demonstrated. Moreover, antagonism of GABA and strychnine has not been observed in the spinal cord where strychnine certainly blocks the action of the inhibitory transmitter. On the other hand, recent experiments suggest that, in the cerebral cortex, the actions of GABA parallel those of the inhibitory transmitter more closely than had hitherto been thought[172]. Whether GABA is an inhibitory transmitter substance in the vertebrate nervous system remains an open question.

An alternative possibility is that GABA regulates the overall excitability of the central nervous system. Evidence that this might be so was provided by some early observations concerning the actions of the convulsant hydrazides. These substances (among which are included isonicotinic acid hydrazide, thiosemicarbazide and thiocarbohydrazide) cause, in experimental animals, spontaneous convulsions or an increased susceptibility to audiogenic

and photically-induced seizures. A tendency to convulsions has also been noted in tuberculous patients who have received therapeutic doses of isonicotinic hydrazide over long periods. The convulsant hydrazides cause a loss of pyridoxine from the body and hydrazide convulsions in experimental animals are associated with a large reduction in the brain's GABA content[173].

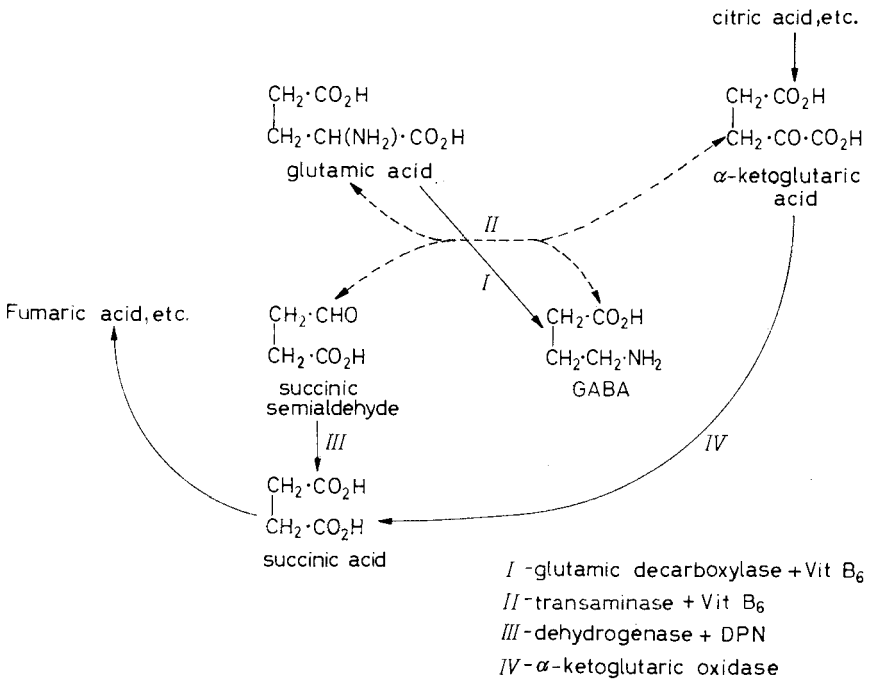


Figure 5.4. The relationship of GABA to the tricarboxylic acid cycle

Hydrazide convulsions can be prevented by the administration of pyridoxine[156] and also by hydroxylamine[174], which inhibits GABA-α-ketoglutaric transaminase. Other compounds, for example penicillamine, which cause pyridoxine deficiency also produce convulsions and hydroxylamine will protect animals against agents, for example leptazol, which do not themselves depress the brain GABA content. These observations seem to indicate a relationship between GABA deficiency and seizure susceptibility but more detailed studies have shown that the relationship is less simple than first appears. Animals given both thiosemicarbazide and hydroxylamine, for instance, still show susceptibility to seizures, though the brain GABA content is higher than normal[156]. Moreover, in a study of regional differences in GABA levels and seizure thresholds, it was found that the most susceptible area of the brain (the hippocampus) had the highest GABA content[175].

Some recent observations have underlined the necessity of interpreting with caution the results of experiments which have sought to modify GABA metabolism. Animals habituated to, and then withdrawn from, barbiturate drugs, develop (as do human beings) spontaneous convulsions or an increased susceptibility to audiogenic seizures[176]. It was shown by Essig that these

convulsions can be prevented by amino-oxyacetic acid, an agent which, like hydroxylamine, causes an increase in brain GABA content[177]. It was concluded that barbiturate habituation and withdrawal led to GABA deficiency and that this underlay the increased seizure susceptibility. However, there is a dissociation between the prophylactic activity of amino-oxyacetic acid and its effect on the GABA system: convulsions can occur in the presence of an elevated brain GABA content and no change in the latter was observed at any stage during the process of habituation to or withdrawal from the barbiturates[178]. Amino-oxyacetic acid confers no protection against alcohol-withdrawal convulsions in rats, though it still causes the accumulation of GABA in the brain in this condition[179].

The functional role of GABA remains, therefore, something of a mystery. There are difficulties in the way of accepting it as the inhibitory synaptic transmitter and it does not seem to be an obvious determinant of cerebral excitability. On the other hand, it must be recalled that brain GABA (which has a general depressant action) stands in a metabolically unique relationship with glutamic acid (which has a general excitatory action on neurones) and it is tempting to suggest that the relative amounts of glutamic acid and GABA in the brain determine (perhaps in a rather more complex way than by the simple ratio of their molar concentrations) its excitability. Such a mechanism might enable the overall cerebral excitability to be influenced by, or to influence, general brain metabolism. It would justify the classification of glutamic acid and GABA with the neuromodulators.

Amino Acids

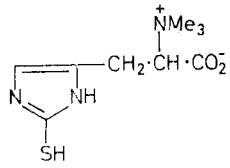
The introduction of the technique of micro-iontophoresis made possible the large scale screening of potential transmitter substances and among the naturally-occurring excitatory compounds thus discovered were the acidic amino acids, notably glutamic acid[180]. In molar concentrations as low as 10^{-15}, glutamate stimulates many cells throughout the central nervous system, increasing membrane permeability in the same way as does an excitatory transmitter. Its action is not unselective: the Betz cells of the cerebral cortex have a high threshold for glutamate excitation and neurones of the olfactory bulb seem to be quite unresponsive. The physiological significance of these observations is not yet clear, particularly since other amino acids, some of which occur in a free state in brain, are also effective. Aspartic, cysteic and *N*-methyl-L-aspartic acids are almost as active as glutamic acid and homocysteic and *N*-methyl-D-aspartic acids are more active[105,181]. It is clear, however, that the physiology of the free amino acids deserves investigation. Reference has already been made to the possibility that glutamic acid and GABA might together be involved in the regulation of cerebral excitability.

Ergothioneine

Crossland and Mitchell[182] argued that, since the cerebellum has a low acetylcholine content, it might be a richer source of non-cholinergic transmitter substances than the rest of the brain. They therefore studied the action of crude extracts of the cerebellum, and of other parts of the brain, on the electrical activity of the cerebellum of the decerebrate rabbit. These

experiments made it clear that cerebellar extracts contained an excitatory material not present in quantity in other large subdivisions of the brain and not identical with any known constituent of brain extracts. This material, provisionally called the cerebellar factor, was also capable of augmenting the electrical activity of the cerebral cortex and of potentiating some spinal reflexes. Its action on the electrical activity of the cerebral and cerebellar cortex was similar to that of acetylcholine but it had little or no action on any of the wide range of smooth muscle preparations which respond to acetylcholine and the other neurohumoral substances. The cerebellar factor was later found in other restricted areas of the central nervous system containing no acetylcholine, notably the optic nerves[183].

Recently the cerebellar factor has been identified as ergothioneine (*IV*), the betaine of thiolhistidine.



(IV) ergothioneine

Ergothioneine has been found in the cerebellum of all mammalian species so far examined. These include the mouse, rat, guinea-pig, rabbit, cat and sheep. In all of them, the ergothioneine content of the cerebellum (which ranges among the different species from 3 to 10 μg per gram) is several times higher than that of the cerebral hemispheres (less than 0·1–1·6 μg/g). Optic nerve extracts have been examined only in the rabbit: they contained large amounts of ergothioneine (about 30 μg/g) in contrast to the dorsal columns of the cord, another acetylcholine-free tract of fibres, in which ergothioneine could not be detected at all[184].

Ergothioneine has been known since 1909 when it was extracted from ergot by Tanret[185]. It is a matter of considerable interest that ergot is also a natural source of acetylcholine and histamine. The most complete study of ergothioneine has been made by Melville, whose comprehensive review[186] is recommended to those requiring more complete information on the subject. Melville was unable to ascribe any physiological function to ergothioneine notwithstanding its wide distribution in peripheral tissues notably the erythrocytes, the liver and the kidneys. Previous work had suggested that ergothioneine was not present in the brain, but this conclusion probably arose because whole brain extracts were studied. The overall ergothioneine content of brain is quite low in comparison with other tissues containing it.

Since the identity of the cerebellar factor was announced, it has been reported that ergothioneine has little action on cerebellar neurones and none on cortical cells[187]. However, it is not yet clear how much ergothioneine reached the neurones in these iontophoretic applications. Recent experiments have shown that ergothioneine stimulates some brain stem neurones when it is applied by micro injection[187a].

The discovery of ergothioneine in the central nervous system is recent

and much more work is needed before any conclusion can be reached concerning its central functions. Its occurrence in quantity in the optic nerves suggests that its functions might be profitably studied in the lateral geniculate body, where the optic nerves synapse with the next neurones in the visual pathway.

Other Compounds

Sensory nerves contain no acetylcholine and the chemical mediator involved in the simplest unit of central nervous activity, the monosynaptic reflex, is not known. For this reason, some considerable attention has been paid to the pharmacological activity of extracts of sensory nerves, which have been shown to contain three substances of potential neuropharmacological significance—ATP, substance *P* (a polypeptide) and histamine. They have been reviewed at length elsewhere[188] and little need be added here, since it now seems likely that neither ATP nor substance *P* will be shown to have neurohumoral functions. However, some psychotropic drugs have anti-histamine activity and a brief statement is therefore necessary concerning the present status of histamine.

So far as its possible central functions are concerned, histamine remains an enigma. It is present in brain, though in smaller quantities than is often reported. The largest amounts of histamine are found in the hypothalamus[189] and the optic nerves[190], but its gross distribution is fairly regular in the rest of the brain. It has been shown to have a stimulant action on the electrical activity of the cerebellum[182,191], though it is not clear whether this effect is due to a direct action on cerebellar neurones. It has proved impossible to confirm[192,193] earlier reports[194] that histamine is present in the cerebellum in larger quantities than in the cerebral hemispheres. Reports that the histamine content of brain is influenced by drugs need to be carefully examined in the light of the assay method used, for the recently introduced and widely used fluorimetric method[195] is much less specific than the biological method[192]. The catalepsy following bulbocapnine administration was reported to be associated with an increase in the histamine content of brain[196], an interesting finding in view of the frequent development of this condition during the administration of psychotropic drugs. It could not be confirmed when biological assay methods were used[192].

CLASSIFICATION AND PROPERTIES OF THE PSYCHOTROPIC DRUGS

The multiplicity of psychotropic drugs makes some form of classification essential and a large number of different groupings has been proposed. To some extent this reflects the fact that a variety of specialists—psychiatrists, chemists and pharmacologists—are involved in the development of these drugs and are necessarily interested in different aspects of their structure and properties. A more important reason for this plethora of attempts to classify the psychotropic drugs is the fundamental impossibility of achieving a simple classification of compounds which possess an extraordinarily wide variety of pharmacological actions and chemical structure and whose one common attribute—a psychotropic action—is extremely ill-defined. The 'psyche' has neither anatomical location nor physiological properties and

any substance which produces an action on the central nervous system, directly or as a result of peripheral stimulation, is potentially a psychotropic drug. Indeed, the increasing evidence that pharmacologically inert placebos can be as clinically valuable as many so-called psychotropic drugs suggests that, depending on the circumstances of their administration, the personality of the clinician and the psychological state of the patient, all substances may have a psychotropic action. In brief, the present impossibility of classifying even the potent psychotropic drugs in any universally acceptable scheme arises from the lack of any clear correlations between chemical structure, pharmacological activity, behavioural effects on the intact animal and clinical usefulness.

Brodie and his school have suggested a simple *physiological* classification[197] based on the view of Hess[198] that diencephalic function involves the interplay of two opposed systems, the 'trophotropic' inhibitory system and the excitatory 'ergotropic' system. Psychotropic drugs are then classified into those whose action is predominantly trophotropic—because they either stimulate the trophotropic or inhibit the ergotropic system—and those with a predominantly ergotropic action. Since Brodie believes that the activities of the trophotropic and ergotropic systems are mediated by 5-hydroxytryptamine and noradrenaline respectively, his classification also implies a simple pharmacological dichotomy of drug action. This scheme suffers from its simplicity: it ignores the existence of all the other putative neurohumours, it implies that all psychotropic action stems from the diencephalon, and it forces ill-assorted compounds into the same category. A *pharmacological* classification has been proposed. In this scheme, drugs are grouped into known pharmacological categories—antihistamines, central nervous stimulants, and so on. Under such a system, if the categories are too few, drugs with very different central actions are grouped together. If the number of categories is large, individual compounds may have to be included in several groups. A more serious criticism of this pharmacological classification is that it implies a knowledge of the mechanism of drug action where none may exist. Thus, to class a psychotropic drug as an antihistamine compound suggests that its antihistamine action forms the basis of its psychotropic effectiveness, though there is little evidence that histamine is critically involved in the regulation of central nervous activity. A *psychological* classification has been put forward by Eysenck[199], but even the most detailed drug profile based on the measurement of psychological variables in normal individuals may be of little value in predicting the therapeutic value of the drug in the psychotic or neurotic patient. An exclusively *chemical* classification suffers from the disadvantage, as later examples will show, that minor modifications of chemical structure may produce major alterations in properties. On the other hand, experience has shown that a chemical subgrouping within major functional divisions is valuable to all types of worker in the field, even though this may result in the appearance of the same type of structure under different functional headings.

Drugs are used to treat the sick human being and there are clear theoretical advantages in adopting one of the several *clinical* classifications that have been put forward. In the lists that follow, psychotropic drugs are grouped according to their clinical effects and the major groups are subdivided into

chemical categories. The scheme is similar in its main essentials to that recommended by the World Health Organization but the number of categories is reduced in order to prevent the drawing of distinctions where none exist. Readers requiring a more extensive subdivision more closely based on the WHO proposals are referred to the excellent review by Shepherd and Wing[200].

The suggested classification (see also Delay[201]) is as follows:

(1) Depressants or psycholeptics (tranquillizers and sedatives)
  (*a*) the phenothiazine derivatives
  (*b*) reserpine and related compounds
  (*c*) diphenylmethane derivatives
  (*d*) the barbiturates
  (*e*) other compounds

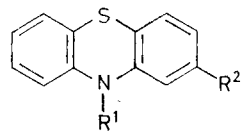
(2) Antidepressant drugs, psychic energizers or thymoleptics
  (*a*) the monoamine oxidase inhibitors
  (*b*) imipramine and other phenothiazine-like compounds
  (*c*) amphetamine-like compounds

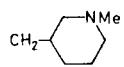
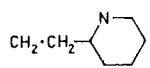
(3) Psychotomimetic drugs

Depressants, Tranquillizers and Sedatives

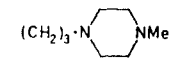
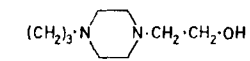
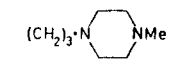
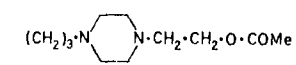
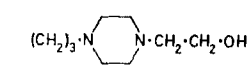
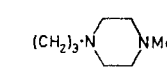
With the introduction of chlorpromazine as a psychotherapeutic agent in 1953 the term 'tranquillizer' was also coined to describe the action of compounds which, like chlorpromazine, have a calming effect on the agitated patient without noticeably depressing his level of consciousness. The distinction between tranquillizers and sedatives (which in large doses do depress the level of consciousness) was made at a time when the importance of the reticular system in the control of consciousness was becoming generally acknowledged, and it seemed at first that the different effects of tranquillizers and sedatives might reflect a real difference in their loci of action, tranquillizers sparing and hypnotics depressing the neurones of the reticular system. It is true that chlorpromazine blocks the arousal response to sensory nerve stimulation at a dose level which produces minimal depression of the reticular response to direct electrical stimulation[202,203] but this property is not common to all tranquillizers. Moreover the depression of the reticular system brought about by chlorpromazine is sufficient to produce the drowsiness which is a well recognized accompaniment of chlorpromazine therapy. The actions of reserpine on the reticular system seem to be predominantly excitatory, notwithstanding an associated behavioural depression[204,205]. Thus reserpine and chlorpromazine have essentially different electrogenic actions though both are classified as tranquillizers, while the barbiturates, which are placed in a different category, have some of the actions shown by chlorpromazine. None of the other reported differences between tranquillizers and hypnotics can be regarded as absolute. Tranquillizers, it is sometimes said, never produce excitement and are not drugs of addiction. The production of catatonia has been regarded as a characteristic feature of tranquillizer action not shared by the hypnotics. Yet some of the phenothiazine tranquillizers do cause restlessness, there are well-authenticated reports of addiction to meprobamate[206] and morphine produces a definite

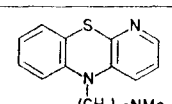
Table 5.1. Some phenothiazine derivatives



Approved name	*Proprietary name*	R^1	R^2
		Compounds with a dialkylamino group in the side chain	
Major Tranquillizers			
Chlorpromazine	Largactil	$(CH_2)_3 \cdot NMe_2$	Cl
Triflupromazine, Flupromazine	Vesprin, Vespral	$(CH_2)_3 \cdot NMe_2$	CF_3
Methoxypromazine	Tentone	$(CH_2)_3 \cdot NMe_2$	OMe
Acepromazine	Notensil, Plegicil	$(CH_2)_3 \cdot NMe_2$	COMe
Methotrimeprazine	Veractil, Neurocil, Nozinan	$CH_2 \cdot CHMe \cdot CH_2 \cdot NMe_2$	OMe
Levopromazine			
Hypnotic			
Propiomazine	Indorm, Dorevane, Largon	$CH_2 \cdot CHMe \cdot NMe_2$	COEt
Minor Tranquillizers			
Promazine	Sparine	$(CH_2)_3 \cdot NMe_2$	H
Promethazine	Phenergan	$CH_2 \cdot CHMe \cdot NMe_2$	H
Ethopropazine, Profenamine	Lysivane, Parsidol	$CH_2CHMe \cdot NEt_2$	H
		Compounds with piperidine ring in the side chain	
Major Tranquillizers			
Pecazine, Mepazine	Pacatal		H
Thioridazine	Melleril		SMe

Compounds with piperazine ring in the side chain

Trifluoperazine	Stelazine		CF_3
Perphenazine	Fentazin, Trilafon		Cl
Prochlorperazine	Stemetil, Compazine		Cl
Thiopropazate	Dartal, Dartalan		Cl
Fluphenazine	Permitil, Moditen, Prolixin		CF_3
Thioproperazine	Majeptil		$SO_2 \cdot NMe_2$

Prothipendyl (Dominal, Phrenotropin, Tolnate) is 10-(3-dimethylaminopropyl)-1-azaphenothiazine 

cataleptic syndrome. Tranquillization is essentially a subjective state and it is questionable whether, as far as the patient is concerned, it is possible to distinguish the calming effect of the tranquillizers from that produced by frank hypnotics such as the barbiturates.

While it is sometimes useful to place in a special category those psycholeptics which, even in large doses, produce relatively little clouding of consciousness, it is clear that there is no real case for a formal separation of the tranquillizers from the hypno-sedatives and it is suggested that attempts to do so are more likely to hinder than to promote a full understanding of the mode of action of psychotropic drugs. Others have suggested on clinical grounds that the distinction between calming and sedation is invalid[207,208].

The unsatisfactory nature of tranquillizer as a descriptive term is underlined by the large number of synonyms that have been coined. These include, among others, ataractic, antiphobic, pacific calmative and peace pill. None seems to have much to recommend it and, so far as it is necessary to refer to these drugs as a group, the term tranquillizer is at least hallowed by use.

The major tranquillizers (drugs of value in the treatment of the severely agitated psychotic patient) are often separated from the minor tranquillizers which are of value only in neurosis. Since this is a difference of degree rather than of kind and since some of the minor and major tranquillizers have the same general structure, this division is not formally made in the present classification but the clinical usefulness of the individual compounds is indicated in the appropriate place.

The phenothiazine derivatives

The structure and names of some of these compounds are listed in *Table 5.1* and a brief account of the pharmacological actions follow.

Chlorpromazine, the first and still most widely-used phenothiazine tranquillizer, is characterized by the variety of its biochemical and pharmacological actions. It has some anti-adrenaline and anti-acetylcholine actions and weaker antihistamine and anti-5-hydroxytryptamine effects[209,210]. Particularly in lower doses, it produces catalepsy in animals and the Parkinsonian syndrome is a recognized side effect of chlorpromazine therapy. Larger doses of chlorpromazine produce flaccidity and weakness. It has an anti-emetic action due to its depressing the chemoceptor trigger zone[211], it produces hypothermia[209], reduces the 'sham rage' reaction in decorticate cats[212] and may inhibit the release of ACTH induced by stress[213,214]. It has some parasympathomimetic effects and causes vasodilatation which is due partly to a peripheral antagonism of noradrenaline and partly to a direct action on the vasomotor centre[209]. In the intact animal it suppresses conditioned avoidance behaviour[215,216].

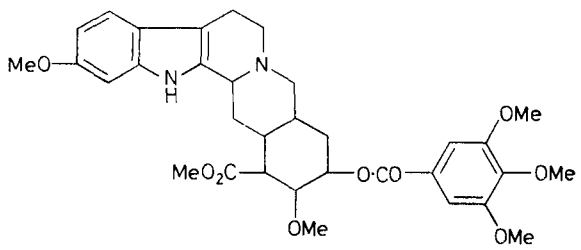
Human beings under the influence of chlorpromazine are apathetic and may fall into a sleep from which, however, they can be easily roused. It seems to have some analgesic effect[209] (though not all workers have been able to confirm this finding[217]) and it potentiates the action of hypnotics and anaesthetics[209,217,218]. On the other hand, restlessness has also been reported to occur during chlorpromazine treatment. Apart from extrapyramidal symptoms the most common side effect of chlorpromazine therapy is obesity. It probably remains the drug of choice for the treatment of schizophrenia.

The other phenothiazine compounds differ from chlorpromazine in the relative intensity of the several properties mentioned above. Thus the piperazine substituted derivatives produce more intense catalepsy and are more powerfully anti-emetic than chlorpromazine itself. Promethazine has powerful antihistamine and anti-emetic actions. Ethopropazine has a marked antiparasympathetic action and so far from causing extrapyramidal symptoms, it is actually used in the treatment of Parkinsonism. Trifluoperazine is much more potent than chlorpromazine. Propiomazine is a true hypnotic, though it also possesses in varying degrees the characteristic properties of the other phenothiazine drugs. The fact that propiomazine is a hypnotic adds weight to the conclusion that there is no real difference between the activity of tranquillizers and hypnotics and that the depressant drugs differ from one another only in the relative intensity of their actions on different functional systems in the brain. Prothipendyl is reported to have both sedative and tranquillizing properties.

Complete discussions of the pharmacological actions and clinical usefulness of the phenothiazine derivatives (and of the other classes of drug dealt with in this review) are available elsewhere[219,222].

Reserpine and related compounds[222a]

Reserpine possesses one outstanding pharmacological property: it releases the monoamines from both their central and peripheral storage sites. The other noteworthy property of reserpine is its parasympathomimetic action which leads to hypotension, bradycardia, miosis and hypothermia[223].



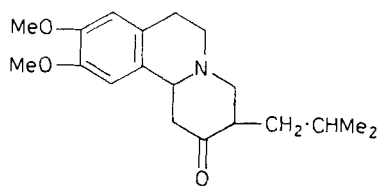
(V) Reserpine

Catalepsy and Parkinsonism have been reported as side actions and the parasympathetic concomitants of these conditions are more marked than they are with phenothiazine therapy. Reserpine, like chlorpromazine, inhibits sham rage reactions[224], makes aggressive animals more tractable[225] and inhibits conditioned avoidance responses[226].

Reserpine was originally used for the treatment of hypertension and the depression which was frequently observed during reserpine therapy suggested that the alkaloid might have psychotherapeutic potentiality. In fact, the root of *Rauwolfia serpentina*, from which reserpine is prepared, had been used in Asia centuries ago for the treatment of manic states. In psychiatrically normal hypertensive patients, the depression induced by reserpine occasionally led to suicide and its use as a hypotensive drug had to be discontinued. Although severe depression occurred much less frequently during the

reserpine treatment of agitated psychotic patients, the risk of suicide was still real and the drug is now rarely used in psychiatric practice. It remains, however, a valuable pharmacological tool. The presence of an indole nucleus is to be noted in view of the possibility that the central actions of reserpine may be associated with disturbances of brain 5-hydroxytryptamine metabolism.

Other reserpine derivatives which have been used in psychiatry include 11-demethoxyreserpine (deserpidine, Harmonyl) and methyl *O*-3,4,5-trimethoxycinnamoyl reserpate (rescinnamine, Anaprel, Moderil). The compound tetrabenazine (Nitoman (*VI*)) which lacks an indole nucleus has similar pharmacological actions to reserpine but its side effects are milder, perhaps because it has few peripheral actions.



(VI) Tetrabenazine

The diphenylmethane derivatives

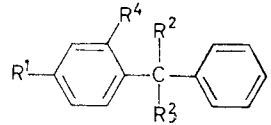
The difficulty of attempting to classify psychotropic drugs on a chemical basis is well illustrated by these compounds. The formulae and pharmacological activities of a number of them are shown in *Table 5.2*. All classes of psychotropic drug from sedatives to central stimulants and psychotomimetics are represented in the table. Of the derivatives listed as minor tranquillizers, only hydroxyzine is of clinical value, though benactyzine deserves mention since it has been used both as a tranquillizer and as an antidepressant!

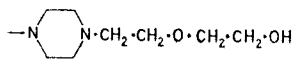
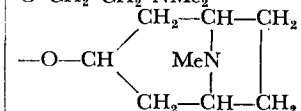
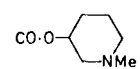
The pharmacological properties of hydroxyzine are quite similar to those of chlorpromazine. It has antihistamine and anti-acetylcholine actions as have, indeed, most of the derivatives listed. Like chlorpromazine, hydroxyzine is anticonvulsive, it potentiates the action of hypnotics, it produces hypothermia and hypotension, it depresses the reticular system and it has some anti-adrenaline action. Extrapyramidal side effects do not, apparently, occur, perhaps because of the drug's rather powerful anti-acetylcholine action which probably also explains such side effects as dryness of the mouth and the occasional occurrence of central excitation. Epileptiform seizures have also been seen during hydroxyzine therapy. The fact that anti-acetylcholine and antihistamine activity is found with many of the diphenylmethane derivatives, whether depressant or excitatory, suggests that the tranquillizing effect of hydroxyzine cannot be due to its antagonizing the action of acetylcholine or histamine.

The barbiturates

Among the barbiturates are found anaesthetics, anticonvulsants, hypnotics and sedatives. They thus have a wider field of usefulness than drugs which

Table 5.2. Some diphenylmethane derivatives



Approved Name	*Proprietary Name*	R^1	R^2	R^3	R^4
Minor tranquillizers					
Hydroxyzine	Atarax, Vistanil	Cl	H		H
Azacyclonol	Frenquel	H	H		H
Captodiamine	Suvren, Covatil	SBu	H	$S \cdot CH_2 \cdot NMe_2$	H
Benactyzine	Suavitil	H	HO	$CO \cdot O \cdot CH_2 \cdot CH_2 \cdot NEt_2$	H
Phenyltoloxamine		H	H	H	$(R_4 = O \cdot CH_2 \cdot CH_2 \cdot NMe_2)$
Antihistamine					
Diphenhydramine	Benadryl	H	H	$O \cdot CH_2 \cdot CH_2 \cdot NMe_2$	H
Anti-Parkinsonian					
Orphenadrine	Disipal, Norflex	H	H	$O \cdot CH_2 \cdot CH_2 \cdot NMe_2$	$(R_4 = Me)$
Chlorphenoxamine	Phenoxane, Clorevan	Cl	Me	$O \cdot CH_2 \cdot CH_2 \cdot NMe_2$	H
Benztropine	Cogentin	H	H		H
Central stimulant					
Pipradrol	Meratran	H	HO		H
Psychotomimetic					
N-methyl-3-piperidyl benzilate	—	H	HO		H

are used specifically for their psychotropic action, and a detailed survey of their properties, structure and uses would involve the discussion of topics far outside the subject of this review. They must, however, be mentioned for they have figured prominently among the drugs used in the treatment of psychiatric conditions. Restlessness by day and insomnia by night are among the more common symptoms of many neurotic and psychotic illnesses and many of the hypnotics are useful, in smaller doses, as sedatives. Such compounds (which include the barbiturates) are sometimes classed as 'hypno-sedatives' to distinguish them from those sedatives which cause only muscular relaxation in higher doses, the 'tranquillo-sedatives'. Since the introduction of the newer tranquillizers, barbiturates have been less popular as sedatives (though they are still widely used as hypnotics) but they still have their place in the treatment of the severely disturbed patient. One well-controlled investigation, indeed, led to the conclusion that for the treatment of neurotic patients, amylobarbitone (Amytal) was a more effective drug than chlorpromazine, meprobamate or reserpine[227].

Amylobarbitone is the barbiturate most widely used for its sedative action. The synergism between barbiturates and some of the phenothiazine derivatives permits their being administered together so that the dose of barbiturate can be reduced. This is an important consideration, in view of the ease with which addiction occurs, particularly to the short-acting compounds such as amylobarbitone.

Some of the physiological and biochemical actions of the barbiturates are similar to those exhibited by chlorpromazine and the other tranquillizers. Thus, the ascending reticular system is inhibited and there is evidence that barbiturates both stimulate[228] and inhibit[229] the hypothalamus. Their greater hypnotic action is probably due to the fact that, like all potential anaesthetics, they cause a general depression of the central nervous system. Like chlorpromazine, some barbiturates uncouple oxidative phosphorylation *in vitro* (p. 301). On the other hand, the barbiturates do not exert any antagonism *in vitro* towards histamine, 5-hydroxytryptamine, noradrenaline or acetylcholine nor is their administration accompanied by signs of extrapyramidal stimulation.

Other compounds

The names and formulae of some of the psycholeptic compounds which cannot be classified in the major chemical categories so far considered are set out in *Table 5.3*. Meprobamate (*VIII*) was one of the earliest used tranquillizers. It was developed from mephenesin (*VII*), which was introduced as a muscle relaxant and was soon seen to have a sedative action too. In small doses, meprobamate is a sedative. In larger doses, it causes muscle relaxation and has been classed, with similar compounds, as a 'tranquillo-sedative'. Like the barbiturates, it produces no sign of extrapyramidal or central autonomic stimulation. Well-authenticated reports of addiction to meprobamate have appeared[206,230]. Both mephenesin and meprobamate produce muscle relaxation by inhibiting interneurones—and hence polysynaptic reflexes—in the spinal cord. Though not described as a hypnotic, meprobamate has been successfully used in the treatment of insomnia, perhaps because it reduces tension.

Meprobamate exerts a unique combination of actions on the central nervous system. These actions are so much those that would be theoretically expected of a tranquillizer that it is surprising that they are not produced by other depressants of the nervous system and that meprobamate is not a more powerful psychotropic agent. It weakly stimulates the ascending reticular system and does not depress the cortex[231] (thus the level of consciousness is unimpaired) but it does selectively depress the thalamus[232] and it inhibits artificially evoked seizure discharges in the limbic system[233] (thus reducing emotional tension). Although it is possible to specify the physiological systems on which meprobamate acts, it is not yet possible to translate this information into terms of the neurohumoral systems involved.

Mephenesin and meprobamate are substituted diols. Other compounds of the same class with muscle relaxant and sedative properties include phenaglycodol (*IX*) and carisoprodol (*XI*). They have little clinical usefulness.

Methylpentynol (*X*) has similar properties to meprobamate. It finds its main use as an hypnotic but it also reduces fear and apprehension sometimes to the point of producing euphoria. Chlordiazepoxide (*XII*) and diazepam (*XIII*) are muscle relaxants inhibiting polysynaptic reflexes. They also have anticonvulsant properties. The most characteristic action of chlordiazepoxide, however, is its ability to reduce hyperirritability, induced or natural. This effect has been demonstrated in a number of animal species, ranging from mice to tigers[219,234]. Human beings are rather less sensitive but the drug is widely used, especially for conditions in which fear or apprehension is a strong component. It has also been employed in the treatment of alcoholism, particularly for the control of withdrawal symptoms. Chlorprothixene (*XIV*) bears a structural resemblance to chlorpromazine but the phenothiazine nucleus is replaced by thiaxanthene. The most interesting aspects of its pharmacology are that it produces Parkinsonian side effects in the human subject and, on isolated preparations, has strong anti-5-hydroxytryptamine and anti-acetylcholine actions with some antihistamine and antinoradrenaline activity. These features are presumably a consequence of the reappearance of the chlorpromazine configuration. Chlorprothixene has been classed as both a tranquillizer and as a thymoleptic. Clopenthixol (*XV*) has similar properties and uses.

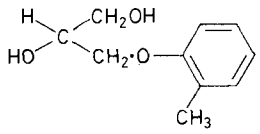
Haloperidol (*XVI*) is a minor tranquillizer which can be regarded as a derivative of GABA; glutethimide (*XVII*) has a formal resemblance to glutamic acid. The remaining compounds (*XVIII–XXI*) in *Table 5.3* are often included among the tranquillizing drugs but they are all hypnotics.

A complete listing of all the psycholeptic drugs would, of course, also include the older depressants and hypnotics such as the bromides, chloral hydrate, and morphine.

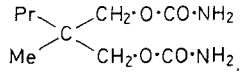
Antidepressant Drugs

Although the drugs included under this general heading have all been used in the treatment of depression, it is important to note that 'depression' is not itself a simple clinical entity and it cannot be easily defined. Depression, associated with emotional excitement and restlessness, is hardly likely to respond to the same agents as does acute melancholia in which all forms of activity, mental and physical, are depressed. Some forms of depression have

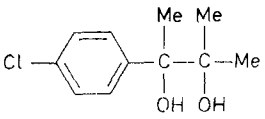
Table 5.3. Miscellaneous psycholeptic drugs



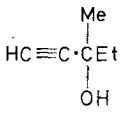
(VII) mephenesin
(Myanesin, Myoten, Prolax, Tolserol, Tolulox, etc.)



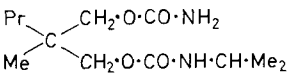
(VIII) meprobamate
(Equanil, Harmonin, Mepavlon, Miltown, Perequil, etc.)



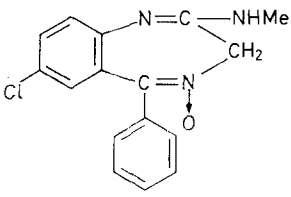
(IX) phenaglycodol
(Ultran)



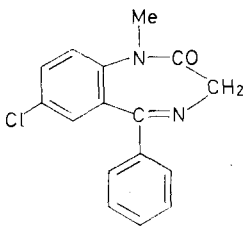
(X) methylpentynol
methylparafynol
(Oblivon)



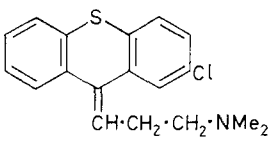
(XI) carisoprodol
(Carisoma, Rela, Soma)



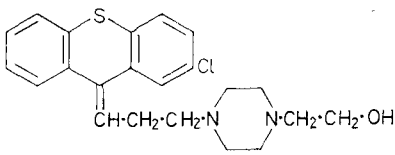
(XII) chlordiazepoxide
methaminodiazepoxide
(Librium)



(XIII) diazepam
(Valium)

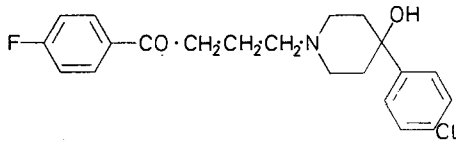


(XIV) chlorprothixene
(Taractan)

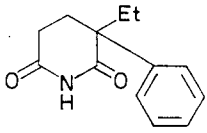


(XV) clopenthixol
(Ciatyl, Sordinol)

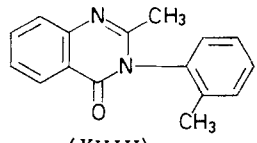
Table 5.3. Miscellaneous psycholeptic drugs (*continued*)



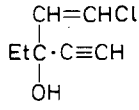
(*XVI*) haloperidol (Serenace)



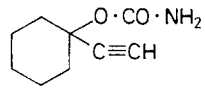
(*XVII*)
glutethimide
(Doriden)



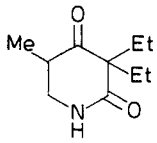
(*XVIII*)
methaqualone
(Melsedin, Tuazole)



(*XIX*) ethchlorvynol
(Arvynol, Serensil)



(*XX*) ethinamate
(Valmid, Valmidate)



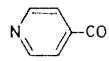
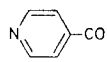
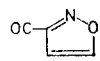
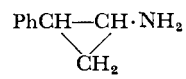
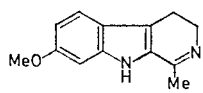
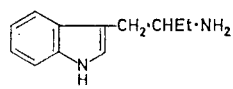
(*XXI*) methyprylone
(Noludar)

been treated with sedatives for more than 2,000 years and it is perhaps not surprising that there is no general agreement as to the drugs which ought properly to be included in the category of antidepressants. The doubts and disputes concerning the relative therapeutic efficacy of the different types of drug cannot, however, be resolved simply by defining more closely the clinical conditions which respond to individual compounds. The introduction of the newer antidepressant drugs was greeted with an enthusiasm which is now noticeably cooling, particularly in Great Britain. There are many who now believe that the drugs are less useful than electric convulsive therapy and some go so far as to assert that the whole of their activity can be explained by a placebo effect.

Monoamine oxidase inhibitors (*Table 5.4*)

The monoamine oxidase inhibitors form yet another group of drugs which were introduced into psychiatry as a result of observations in a different field. It was noticed that tuberculosis patients under treatment with iproniazid frequently became euphoric. The further observation that iproniazid inhibited brain amine oxidase[235] prompted the development of

Table 5.4. Some monoamine oxidase inhibitors

Approved Name	*Proprietary Name*	*Formula*	
		R^1	R^2
		Substituted hydrazines, $R^1NH \cdot NHR^2$	
Iproniazid	Marsilid		$CHMe_2$
Phenelzine	Nardil	$PhCH_2 \cdot CH_2$	H
Pheniprazine	Catron, Cavodil	$PhCH_2 \cdot CHMe$	H
Nialamide	Niamid		$(CH_2)_2 \cdot CO \cdot NH \cdot CH_2Ph$
Isocarboxazide	Marplan	$PhCH_2$	
Phenoxypropazine	Drazine	$PhO \cdot CH_2CHMe$	H
Pivazide	Tersavid	$PhCH_2$	$CO \cdot CMe_3$
Mebanazine	Actomol	PhCHMe	H
	Other compounds		
Tranylcypromine	Parnate		
Harmaline			
Etryptamine, α-Ethyltryptamine	Monase		
Pargyline	Eutonyl	$PhCH_2 \cdot NMe \cdot CH_2 \cdot C \equiv CH$	

other inhibitors. Iproniazid itself inhibits the amine oxidase of liver as effectively as that of brain[235]. This action seems to be related to its hepatotoxic effect, which has led to the virtual abandonment of iproniazid as a therapeutic agent. For a similar reason phenelzine and pheniprazine are no longer used. In addition to its toxic action on the liver, the last-named drug also caused red-green colour blindness, which was sometimes irreversible[236]. Agranulocytosis has occurred in patients treated with etryptamine and this drug too is no longer used. Other side effects of iproniazid treatment included autonomic disturbances (postural hypotension, dry mouth, constipation, diarrhoea, sweating, etc.), dizziness, oedema, muscle weakness and motor restlessness. Liver damage is less likely with the other inhibitors named but cases of severe hepatitis have been reported with all of them and the incidence of the other side effects is as high as with iproniazid itself[237,238]. The occurrence of hypotension in patients treated with iproniazid suggested that

the inhibitors might be used in the treatment of hypertension and pargyline was introduced for this purpose. The complexities attending attempts to provide a pharmacological analysis of the mode of action of therapeutic agents is illustrated by the fact that though reserpine and iproniazid have similar effects on blood pressure, they behave in most other respects as antagonists, though all their principal actions are usually thought of as being mediated through the monoamines.

The most characteristic (but not completely specific[239]) pharmacological action of the monoamine oxidase inhibitors is their ability to reverse reserpine sedation[240] and to potentiate the excitatory effects of 5-hydroxtryptamine administration[109,241]. Given alone, in single doses, they produce no obvious hyperactivity in experimental animals and they may even cause depression. After prolonged administration, sympathetic stimulation and locomotor hyperactivity do occur but these actions are still not so marked as when the inhibitors are administered in conjunction with 5-HTP. The physiological significance of this observation has been discussed earlier (p. 269).

Some of the monoamine oxidase inhibitors prolong the hexobarbitone sleeping time in mice, probably because they inhibit barbiturate breakdown by the liver microsomes[242]. An anticonvulsive action has also been reported[243]. The fact that the hydrazine derivatives interact with pyridoxal phosphate and can thus inhibit GABA formation may explain why some of the inhibitors of this type sometimes have a convulsive action also.

Considerable interest has recently been aroused by reports that patients treated with monoamine oxidase inhibitors may suffer severe hypertensive attacks after taking certain foods, notably cheese[245,246], beans[247] and extracts of yeast[248]. Some of these attacks have proved fatal. The hypertensive crises arise as a result of pressor substances in the offending foods (such as tyramine in cheese) which are absorbed unchanged into the blood stream when intestinal and liver monoamine oxidase is inhibited[249]. Some of the inhibitors (tranylcypromine is an example) also have sympathomimetic actions which will contribute to the hypertensive effect. The administration of sympathomimetic substances—such as adrenaline in a local anaesthetic—to patients treated with monoamine oxidase inhibitor also creates a dangerous situation. The possibility of hypertensive crises clearly constitutes a serious hazard of therapy with these enzyme inhibitors. In many instances their limited effectiveness would not justify the exposure of patients to these hazards.

The monoamine oxidase inhibitors are discussed in more detail by Pletscher, Gay and Zellar[250] and in the monographs referred to earlier[219–222].

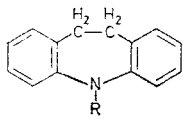
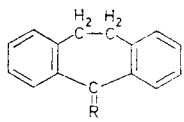
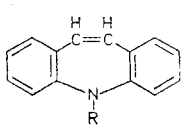
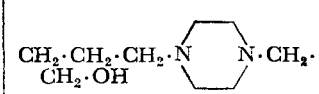
The actions or side effects of a large number of other drugs (antihistamines, local anaesthetics, etc.) may be partly due to or may be modified by monoamine oxidase inhibition—a property which is possessed by a wide variety of other compounds.

Imipramine and similar compounds (*Table 5.5*)

Imipramine is structurally analogous to chlorpromazine but the replacement of sulphur by a —$CH_2 \cdot CH_2$— linkage causes a considerable change in steric and electronic geometry and in pharmacological activity. Like the phenothiazine compounds, imipramine shows some activity against acetylcholine, noradrenaline and histamine[251], and extrapyramidal side effects

have occasionally been observed during imipramine therapy[252,253]. On the other hand, imipramine and the other compounds named in *Table 5.5* also have the ability to antagonize phenothiazine induced catatonia[254]. The other side effects of imipramine are similar to those seen with the monoamine

Table 5.5. Imipramine and related compounds

Approved Name	*Proprietary Name*	*R*
		
Imipramine	Tofranil	$CH_2 \cdot CH_2 \cdot CH_2 \cdot NMe_2$
Trimipramine	Surmontil	$CH_2 \cdot CHMe \cdot CH_2 \cdot NMe_2$
Desipramine	Norpramin Pertofran	$CH_2 \cdot CH_2 \cdot CH_2 \cdot NHMe$
		
Amitriptyline	Elavil, Horizon, Laroxyl, Saroten, Seroten, Tryptanil, Tryptizol	$CH_2 \cdot CH_2 \cdot CH_2 \cdot NMe_2$
Nortriptyline	Aventyl, Allegron	$CH_2 \cdot CH_2 \cdot CH_2 \cdot NHMe$
		
Opipramol	Insidon	

oxidase inhibitors and include hypotension, sweating, dryness of the mouth, dizziness and tremor[255]. Imipramine has recently been reported to have teratogenic activity in rabbits[256] and a few cases of agranulocytosis have been reported in imipramine treated patients[257–259].

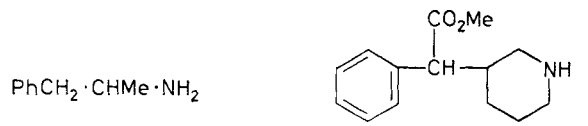
Although imipramine does not inhibit monoamine oxidase, it does reverse the sedative action of reserpine without, however, restoring monoamines to the brain[260]. Imipramine, indeed, can reverse reserpine sedation whether it is given before or after reserpine: the monoamine oxidase inhibitors are effective only when given after reserpine[261]. Like some of the monoamine oxidase inhibitors, imipramine and amitriptyline prolong barbiturate sleeping time and have marked anticonvulsive potency[262,263]. At the same time, they potentiate the action of central stimulants such as amphetamine[264,265]. Amitriptyline inhibits gastric secretion and the possibility of using it in the treatment of peptic ulcer has been explored.

In the otherwise unmedicated animal, imipramine and its related compounds have even fewer behavioural effects than the monoamine oxidase inhibitors. Such changes as have been recorded, using conditioned avoidance and escape techniques, suggest that behaviourally the drugs resemble the tranquillizers[239,263,265]. Chlorpromazine and amitriptyline have similar depressant effects on the electrical activity of the brain, reducing the frequency of the spontaneous rhythms. Amitriptyline has been used for treating patients in whom depression and anxiety occur together.

The clinical effectiveness and pharmacological properties of the other compounds named in *Table 5.5* are broadly similar to those of imipramine and amitriptyline. Imipramine appears to owe its activity to desimipramine into which it is converted in the body. Its effects are consequently slower to show themselves than are those of desimipramine.

Amphetamine and other compounds

Amphetamine (*XXII*) is a central stimulant and many would not classify it with the antidepressant drugs proper. It has, however, been extensively used in the treatment of depression, it produces euphoria and some at least of its actions may be due to inhibition of monoamine oxidase. However, it also inhibits dopamine-β-oxidase, impairs the noradrenaline binding capacity of the brain and has direct sympathomimetic activity. Its classification with the antidepressants seems, therefore, to be justified, but it is not included with the monoamine oxidase inhibitors, since only a small part of its action can be attributed to enzyme inhibition. Amphetamine is a potentially addictive drug and it should be used cautiously and over short periods of time. Other compounds which are used, if at all, only for the treatment of mild depression, include methylphenidate (*XXIII*), pipradol (*Table 5.2*) and deanol (*XXIV*). The last named compound is interesting since it may owe its effectiveness to a stimulant action on acetylcholine synthesis[266,267].



(XXII) amphetamine (Benzedrine)

(XXIII) methylphenidate (Ritalin)

$Me_2N{\cdot}CH_2{\cdot}CH_2{\cdot}OH$

(XXIV) 2-dimethylaminoethanol, deanol (Deaner)

Psychotomimetic Drugs

Psychotomimetic compounds are included here with the psychotropic drugs, as they are in the WHO classification, though their therapeutic usefulness is limited. They are so called because they induce, in normal subjects, changes in mood, perception or behaviour which mimic some of

those seen and experienced in the schizophrenias. The term 'psychotomimetic' has attracted many synonyms: 'hallucinogen' is the most common, but 'schizogen', 'psychotogen', 'phantastica', 'psychosomimetic' and 'psycholytic' (to name only a few) are also used.

It is important to recognize that the resemblance between the drug-induced symptoms in normal subjects and those occurring naturally in psychotic patients is far from complete. Thus although the psychotomimetic drugs are said to be hallucinogenic, this description is not strictly true. Subjects under their influence do experience vivid, often brightly coloured, visual sensations, but these, however bizarre, almost always represent distorted images of some actual object in the visual field. The images are, in fact, usually illusions; hallucinations, which are experienced in schizophrenia, are properly defined as sensory experiences which arise in the absence of any physiological stimulation of the corresponding sense organs. Visual hallucinations can occur in the dark or when the eyes are closed. The rarity of true drug induced hallucinations in the normal subject is probably not simply the reflection of an inherent difference between the responsiveness of the normal and the psychotic individual, for hallucinations are frequently experienced by normal people in experimental situations in which perceptual stimulation has been reduced to a minimum.

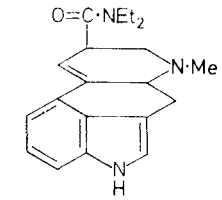
It is usual to classify as psychotomimetic, substances which produce predominantly psychotic effects in low concentrations. Many other drugs (cocaine, amphetamine and atropine, for instance) have psychotomimetic side effects or are effective psychotomimetics in relatively high doses. They are, therefore, not usually grouped with the psychotomimetic drugs, but since the criteria of psychotomimetic action are rather imprecise, differences of opinion do arise concerning the classification of some of the less active substances. The names and formulae of some of the compounds generally recognized as psychotomimetic are set out in *Table 5.6.*

Psychotomimetic drugs are not used as chemotherapeutic agents but lysergic acid diethylamide has, in some instances, proved a valuable adjunct to conventional psychotherapy. It appears to revive repressed memories whose psychogenic influence can thus be recognized and dealt with by the patient and his psychiatrist. The patient, to quote an enthusiastic advocate[268] of lysergic acid therapy, 'accepts himself completely for what he is with a massive reduction in self-conflict and guilt'. In general, however, psychiatrists have been less impressed by the therapeutic potentialities of the psychotomimetic drugs than by the possibility of increasing their knowledge of psychiatric conditions by using the drugs to produce 'model psychoses' in normal subjects who can report on their subjective experiences. The interest of the pharmacologist in these drugs is directed towards establishing the nature of their interaction with humoral mechanisms. Such information should provide valuable clues to the biochemical lesion responsible for mental illness.

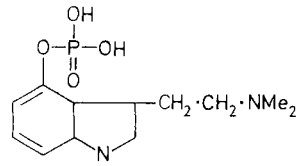
Mescaline is the active principle of the peyote cactus and its psychotomimetic properties were first recognized many centuries ago by primitive South American tribes who used peyote buttons in religious ceremonies. The priestly use of psychotomimetic compounds finds a ready explanation in the heightened sensory awareness and the sensation of 'looking upon

dazzling light and beauty' reported by subjects who have taken the drugs[268]. The effects of mescaline and lysergic acid are, in general, similar, though mescaline does not have the same power of releasing repressed memories as

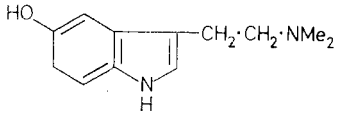
Table 5.6 Psychotomimetic compounds



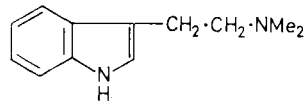
lysergic acid diethylamide
(LSD 25)



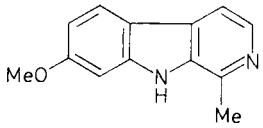
psilocybin



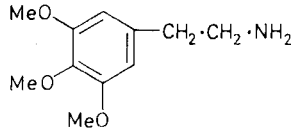
bufotenine



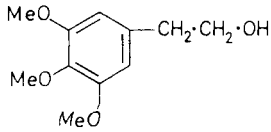
N,N-dimethyltryptamine



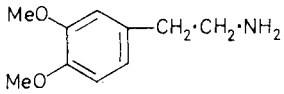
harmine



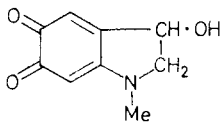
mescaline



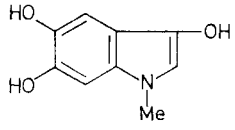
2-(3,4,5-trimethoxyphenyl)-
ethanol



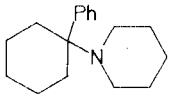
2-(3,4-dimethoxyphenyl)-
ethylamine



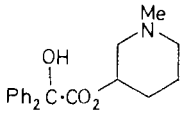
adrenochrome



adrenolutin



phencyclidine
(Sernylan, Sernyl)



N-methyl-3-piperidyl
benzilate

lysergic acid. Lysergic acid, however, tends to produce depressive episodes and sometimes 'delusions of grandeur'. Both of these conditions may lead to suicide.

PSYCHOTROPIC DRUGS AND NEUROHUMORAL PROCESSES

Reserpine, Chlorpromazine and other Psycholeptic Drugs

Reserpine depletes postganglionic sympathetic fibres of their noradrenaline and the peripheral sympathetic responses of animals which have been pretreated with reserpine are those which would be expected to follow loss of sympathetic transmitter[269]. Thus, in adrenalectomized, reserpinized cats, stimulation of the splanchnic nerves does not produce the increase in blood pressure which is seen in non-reserpinized animals. Hexamethonium normally produces hypotension due to ganglionic blockade: in the reserpinized animal it is without effect. Reserpine also causes a loss of adrenaline from the adrenal medulla. Since this adrenaline is liberated into the blood stream, it is not surprising that reserpine causes hyperglycaemia.

Reserpine also causes a loss of noradrenaline from the brain, but it has a similar effect on 5-hydroxytryptamine, dopamine[270], histamine[271] and GABA[272]. Attempts to determine which, if any, of these substances is most critically concerned in reserpine action have led to conflicting hypotheses which cannot yet be satisfactorily resolved.

The view that the central actions of reserpine are due to a 5-hydroxytryptamine deficiency has been vigorously canvassed by Brodie and his colleagues[273,274]. Brodie believes that reserpine interferes with the uptake of monoamines into the storage granules. There is general agreement that this is so, but the rest of Brodie's hypothesis is more controversial. If, so the argument runs, monoamine synthesis is unimpaired but storage is blocked, there should be a continuous release of the newly synthesized compound which, if it is a transmitter, might reasonably be expected to cause a continued stimulation of the appropriate neurones. For reasons which are summarized later, Brodie believes that 5-hydroxytryptamine is the most important of the substances involved in reserpine action, and that the central actions of reserpine are due to stimulation, particularly of diencephalic neurones, by 5-hydroxytryptamine. Since reserpine has a depressant action, it is concluded that serotonin is the transmitter substance of the essentially inhibitory trophotropic system. As has already been discussed, 5-hydroxytryptamine accumulates in the brains of animals fed 5-HTP. This accumulation is accentuated in the presence of monoamine oxidase inhibitors and it is accompanied by signs of behavioural stimulation. A similar situation arises if reserpine is administered in conjunction with a monoamine oxidase inhibitor. If 5-hydroxytryptamine is the transmitter substance at the central synapses of the trophotropic system, these results can only be explained on the assumption that the transmitter has reached such a concentration that it is inhibiting the neurones it normally stimulates, resulting in unopposed activity of the stimulant ergotropic system.

The view that reserpine owes its action to the liberation of 5-hydroxytryptamine rather than noradrenaline is based first on the fact that 5-hydroxytryptamine is more regularly associated with sedation than is noradrenaline release[275–277]. Reserpine and those of its derivatives that have a tranquillizing action release both 5-hydroxytryptamine and noradrenaline but noradrenaline is also released by substances, such as morphine in the

cat, which produce excitement. Moreover, if the central actions of reserpine are to be explained on the same basis as its peripheral action, a considerably greater depletion of noradrenaline would be expected before sedation occurs than the 50 per cent which is actually observed. Finally, the outflow of sympathetic impulses from the brain should decrease if reserpine were producing a deficiency of brain noradrenaline.

Before discussing the pharmacological evidence against Brodie's hypothesis, the reservation prompted by physiological considerations should be noted. It is an undoubted fact that excess of an excitatory substance may cause inhibition. Acetylcholine, for instance, the acknowledged neuromuscular transmitter substance, produces muscular paralysis in a variety of experimental circumstances. It is also true that depression of nervous activity can arise either from stimulation of inhibitory or inhibition of excitatory systems. These facts, as the writer has pointed out elsewhere[89], provide a situation which enables the action of any active drug to be explained in terms of any neurohumoral hypothesis the investigator chooses. In the particular instance now being discussed, further caveats have to be entered. The evidence suggests that the binding of 5-hydroxytryptamine and of noradrenaline is controlled by identical mechanisms and that peripheral neurones are organized in this respect in exactly the same way as central neurones. Yet the peripheral actions of reserpine are consistent with depressed sympathetic function due to transmitter deficiency rather than to continued transmitter release. It would seem reasonable to seek an explanation of the central actions of reserpine similarly in terms of transmitter deficiency. It should also be added that there is no evidence that noradrenaline is involved as a transmitter substance in the neurones of the higher centres of the sympathetic system so that the absence of a decreased sympathetic outflow in reserpine treated animals does not in itself indicate that noradrenaline is not depleted.

No direct evidence has been provided for the view that, notwithstanding a fall in the total brain 5-hydroxytryptamine content, the availability of the 'free' amine is increased. In animals whose brain 5-hydroxytryptamine has been greatly reduced by dietary restriction of tryptophan, reserpine has its usual sedating action[111]. The suggestion that the excitatory actions of 5-HTP are due to the inhibition, by large quantities of 5-hydroxytryptamine, of neurones which are stimulated by the smaller quantities liberated by reserpine is not supported by the observation that 5-HTP is incapable of reversing the action of reserpine[141]. Finally, no consistent correlation has been found between the amount of 5-hydroxytryptamine in brain, or in any of its subcellular fractions, and the extent of behavioural depression.

The time course of noradrenaline loss and recovery in the brain of reserpinized animals is identical with that of 5-hydroxytryptamine and the possibility has to be considered that the central actions of reserpine are attributable to noradrenaline deficiency. Carlsson is the most active protagonist of this view[120,278]. It is rather more acceptable than the 5-hydroxytryptamine hypothesis on theoretical grounds, for it associates depletion of transmitter with impairment of synaptic function and it is consonant with the established peripheral actions of reserpine. It does, of course, assume that noradrenaline is a central transmitter substance. The evidence that this

is so, as has already been discussed, is not yet so convincing as its advocates maintain. Nevertheless, it is profitable to discuss whether reserpine sedation can be explained on the basis of noradrenaline deficiency.

Much of the voluminous experimental evidence which has been adduced to support or to oppose the 5-hydroxytryptamine or the noradrenaline hypotheses consists of attempts to demonstrate that the behavioural effects of drugs are more closely correlated with one or the other monoamine. The results of these investigations are quite conflicting and the most important lesson that can be learnt from them is that conclusions concerning the role of neurohumoral substances, based on the actions of drugs (which are likely to have a multiplicity of chemical actions) should be treated with caution. Thus, while the monoamine oxidase inhibitor *N*-phenylisopropyl-*N'*-isopropylhydrazine reverses reserpine sedation and increases the 5-hydroxytryptamine content of brain[279], isopropylhydrazine increases brain 5-hydroxytryptamine levels but does not reverse the sedation[280]. Although several reports suggest that sedation is more regularly associated with depletion of 5-hydroxytryptamine rather than noradrenaline, it is also true that DOPA (unlike 5-HTP) can reverse reserpine induced depression[141], and the balance of evidence suggests that an explanation of reserpine action should be sought for among its effects on noradrenaline activity. The presence of the indole nucleus in reserpine should not, however, be forgotten.

The actions of α-methylmetatyrosine (α-MMT) and α-methyldopa have been intensively studied recently and they deserve some detailed mention.

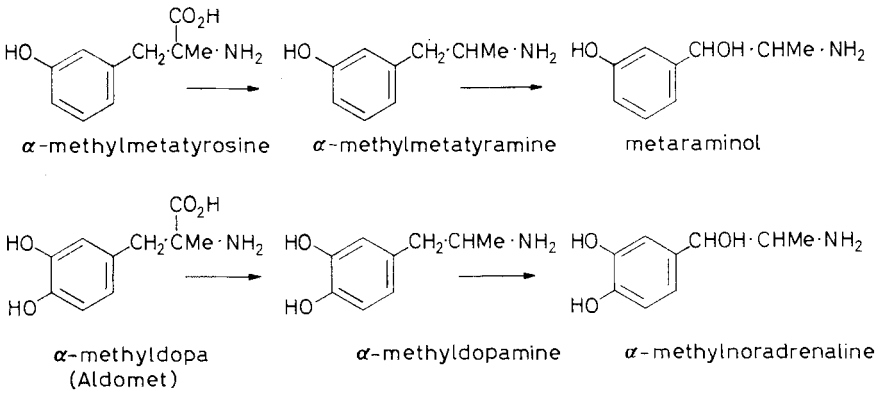


Figure 5.5. α-Methylated derivatives of the catecholamines

They are decarboxylated in the body and their decarboxylated products (*Figure 5.5*) cause a loss of noradrenaline from both peripheral and central neurones[281–283]. 5-Hydroxytryptamine is only slightly affected and it seemed that these compounds might provide a tool for determining the relative importance of 5-hydroxytryptamine and noradrenaline. Unfortunately their use has not resulted in the decisive conclusions originally hoped for.

α-MMT and α-methyldopa cause a loss of up to 97 per cent of the brain's noradrenaline without producing any obvious behavioural change. The first conclusion was that since no overt consequence followed an almost complete loss of noradrenaline, 5-hydroxytryptamine must be the primary determinant

of behaviour. However, these compounds also deplete postganglionic sympathetic fibres of their noradrenaline without affecting sympathetic responses[278,284]. (A recent report suggests that in some experimental conditions a decrease of sympathetic responses can be demonstrated in cats after α-MMT but not after α-methyldopa[285].) It can hardly be argued that therefore noradrenaline is not concerned in the transmission of impulses from sympathetic fibres to their effector organs. Instead, it has been suggested that metaraminol and α-methylnoradrenaline replace noradrenaline, are liberated by sympathetic nerve impulses and thus act as 'false transmitters'. A similar situation might arise in central neurones. An obvious objection to the false transmitter hypothesis is that metaraminol and α-methylnoradrenaline are not very powerful sympathomimetic agents and it is not immediately clear how sympathetic activity can remain completely unaffected when a powerful transmitter is replaced by a weaker agent. Carlsson[278] has provided another possible explanation of this action. There is evidence that noradrenaline is contained in at least two 'pools', a labile pool which is the immediate source of the noradrenaline which is liberated by nerve impulses and a much larger stable pool which acts as a reservoir which replenishes the labile pool when necessary. It is assumed that newly-formed noradrenaline is incorporated into the labile pool and is secondarily transferred to the stable pool. If reserpine prevented the uptake of noradrenaline by the labile pool, it could halt transmission without seriously reducing the total brain noradrenaline content. The decarboxylated derivatives of α-MMT and α-methyldopa, on this view, enter both pools so replacing the noradrenaline. Synthesis of noradrenaline continues and some is incorporated into the labile pool from which it is liberated by nerve impulses in the usual way.

The most recent evidence suggests that the false transmitter hypothesis might, after all, furnish the better explanation of the mode of action of α-MMT. α-Methyl (*p*) tyrosine (α-MT) causes a loss of noradrenaline from the brain and, unlike α-MMT, it produces sedation in a number of animal species. α-MT also inhibits the conditioned avoidance response and this inhibition is correlated, to some extent at least, with a reduction in the noradrenaline content of the brain[285]. It is also clear that the sympathomimetic activities of metaraminol and α-methylnoradrenaline are not so uniformly less than that of noradrenaline as was at first thought; the relative potencies of these compounds depend very much on the species and preparation studied. There is also recent evidence that the replacement of noradrenaline by the false transmitters is accompanied by an increased sensitivity of the effector organs[286]. It should, perhaps, be added that these facts, though welcome to the neuropharmacologist, make it even more difficult to explain the efficacy of α-methyldopa as a hypotensive agent.

Preoccupation with the conflicting claims of noradrenaline and 5-hydroxytryptamine should not be allowed to obscure the fact that reserpine has actions on other neurohumoral systems. It has already been pointed out that reserpine causes a loss of GABA and dopamine from the brain. The case of dopamine is particularly important since compounds such as α-MT and α-MMT reduce the dopamine content of brain, though not to the same extent as the noradrenaline content. Everett and Wiegand measured the 5-hydroxytryptamine, noradrenaline and dopamine content of brain during

altered behavioural activity produced by a variety of agents. They found that dopamine was more closely correlated than was either noradrenaline or 5-hydroxytryptamine with the level of activity[287]. Reserpine has also been reported to increase the acetylcholine[288–290] and (on chronic administration) the substance *P* content of brain[291] and to inhibit brain cholinesterase *in vivo*[292]. Recent experiments have failed to confirm the effect on acetylcholine[47].

Reserpine has a number of biochemical actions. It potentiates and prolongs the elevation of liver diphosphopyridine nucleotides brought about by nicotinamide. This action is shared by reserpine derivatives which have tranquillizing properties but not by those without tranquillizing activity[293]. This effect is thus as characteristic as is monoamine depletion.

Although it has been reported that reserpine increases the amount of ATP in the brain[294], Kirpekar and Lewis[295] have demonstrated that reserpine sedation was accompanied by a decrease in the ATP, and a corresponding increase in the ADP content of brain. The development and reversal of these changes paralleled the behavioural changes.

Chlorpromazine is as powerful a tranquillizer as reserpine but it shares few of the pharmacological and biochemical actions of reserpine. It does not cause a reduction in the noradrenaline and 5-hydroxytryptamine content of brain, though it can block both the increase in brain monoamine concentration brought about by monoamine oxidase inhibitors and the depletion produced by reserpine[296]. In schizophrenic subjects, reserpine treatment, as might be expected, reduces the urinary excretion of 3-methoxy-4-hydroxymandelic acid. Chlorpromazine does not have this action[297]. Its effects on the other neurohumours are small and variable: it has been reported to cause an increase in the acetylcholine content of brain[298] and to inhibit brain cholinesterase[299]. Other workers, however, found that chlorpromazine slightly depressed the acetylcholine content of brain[47]. Chlorpromazine increases the amount of glucose in brain without affecting the phosphorylated intermediates and it has been concluded that it suppresses cerebral glycolysis[300]. However, the increased brain glucose is associated with a reduction in the amount of glycogen in the brain and this glycogen deficiency is more pronounced in adrenal-demedullated animals[301]. The most characteristic biochemical action of chlorpromazine is its ability to uncouple oxidative phosphorylation[302–304], due presumably to its interaction with enzymic SH groups since the uncoupling is reversed by cysteine and glutathione. It is interesting that uncoupling occurs in the cells of the hypothalamus with much lower drug concentrations than are effective in other regions of the brain[305]. There are conflicting reports concerning the effect of chlorpromazine on brain ATP: increases[306], decreases[307] and no change[308] all having been reported. It is, however, well established that chlorpromazine inhibits ATPase activity of brain homogenates but not of powder[309].

Of the other sedatives and tranquillizers, tetrabenazine and related compounds have properties similar to reserpine, including the ability to reduce the monoamine content of brain and phenothiazine derivatives with tranquillizing actions uncouple oxidative phosphorylation and inhibit ATPase activity to an extent roughly corresponding to their tranquillizing

potency[310]. Some of the non-phenothiazine tranquillizers (meprobamate, hydroxyzine and benactyzine) have similar effects. Kaul and Lewis[311] found that a range of minor tranquillizers depressed the ATP:ADP ratio of rat brain in the same way as reserpine. Rather high drug doses were used in these experiments. Meprobamate even at a dose level of 200 mg/kg (the therapeutic dose in man is of the order of 10 mg/kg) had no effect on the ratio, while benactyzine elevated it. It should also be noted that, notwithstanding the high doses employed, the depression of the ATP level was never more than about 0·4 μM/g of brain or about 17 per cent of the total brain complement. Some barbiturates also cause an uncoupling of oxidative phosphorylation and many reduce oxygen consumption by brain *in vitro*, particularly if its respiration is stimulated electrically or by increasing the potassium concentration of the incubation medium. These changes should depress ATP formation but it has never proved possible to demonstrate a reduction in the ATP content of brain *in vivo* during barbiturate depression or anaesthesia. The biochemical effects of a large number of drugs which have an action on the central nervous system have been extensively reviewed by Decsi[312].

It seems, therefore, that central nervous depressants may have one of two characteristic actions: they either deplete the brain of its monoamines or they uncouple oxidative phosphorylation. Some depressants have neither of these actions, none has both, though as has been seen, reserpine and the phenothiazine derivatives slightly reduce the ATP content of brain. Not only depressant drugs uncouple oxidative phosphorylation; the antidepressant imipramine for instance, is much more active in this respect than chlorpromazine[313]. It is possible, however, as Decsi has pointed out, that the anatomical site of maximal uncoupling is important in determining the pharmacological consequences of the biochemical lesion.

It is not easy to see the relationship between depletion of monoamines (or other neurohumours) and uncoupling of oxidative phosphorylation. Acetylcholine synthesis and monoamine binding both require ATP, and Quastel has long argued that anaesthetic action might arise from the blocking of acetylcholine synthesis due to ATP lack consequent on the uncoupling. Unfortunately, there is no evidence that *in vivo* the synthesis of acetylcholine or the availability of ATP is diminished during anaesthesia. The ATP deficiency reported by Lewis does not seem to be sufficient, in view of the large doses of drug required to elicit it, to have profound effects on amine binding or central nervous metabolism, nor is it regularly associated with a detectable reduction in the acetylcholine content of brain. Spirtes and Guth[314] have suggested that the protean actions of the phenothiazines might be explained by their actions on membrane permeability, a stabilization of the membrane leading to inhibition of transmitter release. A number of the actions of chlorpromazine, including the prevention of both reserpine-induced depression and monoamine-induced elevation of brain monoamines can be explained in this way, but a more detailed comparative study of all the depressant drugs is needed before the hypothesis can be properly assessed. From what has been said concerning the lack of a precise knowledge of the central function of the monoamines, it is clear that it is not yet possible to give a satisfactory explanation of the mechanism of tranquillizer and

sedative action. A complete understanding of the mode of action of the several classes of depressant drug must await a more complete knowledge of the anatomical structures, the physiological mechanisms and the synaptic transmitters involved in the control of consciousness and behaviour.

Antidepressants

An explanation of the mode of action of the monoamine oxidase inhibitors in terms of their intervention in central transmission processes is difficult for several reasons. Monoamine oxidase is widely distributed in the body and some of the consequences of its inhibition may arise peripherally: in the brain the enzyme participates in the inactivation of at least three substances (noradrenaline, dopamine and 5-hydroxytryptamine). Not all monoamine oxidase inhibitors have antidepressant activity but those that have may owe at least part of their activity to an action other than enzyme inhibition.

Although the inactivation of 5-hydroxytryptamine is more exclusively dependent on monoamine oxidase than is that of noradrenaline, the behavioural changes which follow the administration of monoamine oxidase inhibitors are more closely associated with elevation of the noradrenaline content of brain. Thus, while a single dose of iproniazid increased the 5-hydroxytryptamine content of the brain of the rabbit, little behavioural change occurred and the noradrenaline content remained virtually unaltered[312]. With repeated doses of iproniazid, the brain noradrenaline content rose and signs of sympathetic stimulation and increased motor activity appeared as the maximum level was reached[313]. Conversely, following cessation of iproniazid treatment, the amount of noradrenaline in the brain returned to its pretreatment value concurrently with the disappearance of the signs of behavioural stimulation. The 5-hydroxytryptamine content remained elevated. In the rat, administration of iproniazid or tranylcypromine caused a transient rise in the brain noradrenaline content and a longer lasting augmentation of the 5-hydroxytryptamine concentration[314]; motor activity was unaffected by the treatment. Monoamine oxidase inhibitors reverse reserpine-induced depression which itself seems to be more closely linked with noradrenaline than with 5-hydroxytryptamine depression. Some monoamine oxidase inhibitors[315] (but not, apparently, all[316]) produce an increase in the dopamine content of brain which also follows a similar time course to that of the behavioural stimulation.

The monoamine oxidase inhibitors have other actions: the hydrazides inhibit pyridoxal-dependent enzymes and thus lower the γ-aminobutyric acid content of brain. There is also evidence that they prevent the release of noradrenaline from brain binding sites[317].

Although the available evidence suggests that the antidepressant action of the monoamine oxidase inhibitors stems from their ability to increase the amount of noradrenaline in the brain, it should be remembered that many other compounds, including some depressants, also possess some ability to inhibit monoamine oxidase. Moreover, orally administered DOPA which, like the enzyme inhibitors, should also increase both the dopamine and the noradrenaline content of brain, is quite ineffective in relieving depression[318], though, as pointed out earlier, it does reverse sedation due to reserpine. Even if the effects of monoamine oxidase inhibitors are the consequence of

increased noradrenaline or dopamine storage, the relationship between the pharmacological and the behavioural changes will remain unexplained until more is known of the physiological role of the monoamines. Many of the reservations concerning the hypothesis that tranquillizer action is due to monoamine depletion apply equally to the complementary hypothesis that antidepressant activity is due to monoamine excess.

Apart from the properties conferred by the phenothiazine-like structure, the pharmacological actions of imipramine and related compounds, which are not strong inhibitors of monoamine oxidase and which do not increase the monoamine content of brain, are very similar to those of the monoamine oxidase inhibitors themselves. Several groups of workers have shown that imipramine reduces the excretion of 3-methoxy-4-hydroxymandelic acid, the principal metabolite of adrenaline and noradrenaline[318–320]. This may be due to a change in the intracellular distribution of noradrenaline, more being taken into the storage granules and being thus spared from destruction. Since monoamine oxidase exerts its primary action on the processes concerned with monoamine storage, imipramine administration might effectively have the same central consequences as inhibition of the enzyme. This explanation recalls that put forward earlier (p. 301) to explain the similar therapeutic efficacy of two compounds—chlorpromazine and reserpine—with different pharmacological properties.

Neuropharmacology of Parkinson's Disease

There is an association between Parkinson's disease and brain dopamine deficiency. The urinary excretion of dopamine and its metabolites is depressed in Parkinsonian patients[301,322] and their brain dopamine is reduced, the caudate nucleus and putamen often containing less than 10 per cent of their normal complement[323,324]. In a subject affected on one side of the body only, it was found that dopamine depletion was more severe in the basal ganglia of the contralateral side. Noradrenaline and 5-hydroxytryptamine are also lost from the brain in Parkinsonian patients to a considerably smaller extent than dopamine. The akinesia and rigidity, but not the tremor, of Parkinsonism can be relieved by L-DOPA administration[325]; however, 5-HTP is reported to be relatively ineffective in this respect[326].

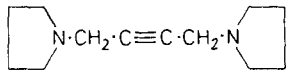
Although the several varieties of extrapyramidal disease show qualitatively similar symptomatologies and although there seems to be no distinct pathological lesion in Parkinson's disease (p. 254), the biochemical lesion is apparently specific. Disturbances of dopamine metabolism have not, for instance, been detected in cases of Huntington's chorea[327] or of Wilson's disease[328], though the pathological lesions may be severe.

Reserpine depletes the brain of dopamine and it is interesting that it produces only a slight increase in extrapyramidal symptoms in cases of severe Parkinsonism although it retains its tranquillizing activity[324]. There is evidence also that, as in reserpinized animals, monoamine oxidase inhibitors do not increase the amount of dopamine in the brain of Parkinsonian patients, though accumulation of noradrenaline does occur. This has led some to suggest that in Parkinson's disease there is a reserpine-like principle in brain which interferes with the storage of dopamine.

Although the evidence so far presented suggests that Parkinson's disease

might be due to a central dopamine deficiency, the drugs commonly used for its treatment are not related to dopamine but to acetylcholine. For many years the most widely used therapeutic agents were atropine and hyoscine. Some still regard hyoscine as the drug of choice, but it has been largely replaced by synthetic compounds (*Table 5.7*). All these compounds except methixene share with atropine and hyoscine the property of antagonizing the actions of acetylcholine. In their central actions they may be either depressant (like hyoscine) or excitatory (like atropine) and some also have antihistamine activity. Unlike DOPA, all these compounds can reduce the tremor as well as the rigidity and akinesia of Parkinson's disease.

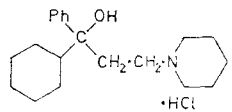
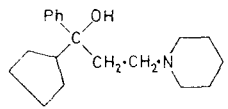
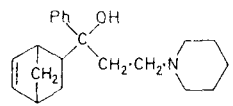
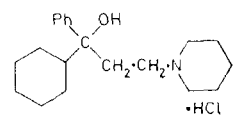
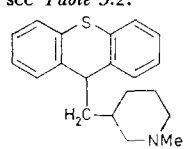
The screening of compounds for possible anti-Parkinsonian activity is best performed with the aid of tremorine (*XXV*) which produces, in experimental animals, the Parkinsonian triad of asynergia, tremor and akinesia, together with hypothermia and intense parasympathetic stimulation. Knowledge of the mode of action of tremorine should therefore contribute towards an understanding of the biochemical aetiology of Parkinsonism, though in view of the complexities of extrapyramidal disease, care has to be taken not to imply too close a parallelism between the acute condition induced by tremorine and the chronic and progressive state produced by the degenerative lesions of Parkinson's disease. Hypothermia, for instance, is not a feature of extrapyramidal disease (though anti-Parkinsonian drugs do effectively prevent the hypothermia due to tremorine[329]) and there is evidence that tremorine has some action on neurones in the spinal cord[330] and hypothalamus[338], a part of the central nervous system spared in Parkinson's disease.



(XXV) Tremorine

Tremorine does not affect the dopamine or the 5-hydroxytryptamine content of mouse brain[331]. It causes a small but statistically significant reduction in brain noradrenaline concentration in this species[331] and an increased 5-hydroxytryptamine content has been reported in rats[332]. Its most marked influence, however, is on the acetylcholine system. Among anti-Parkinsonian drugs, there is a good correlation between antitremorine activity and potency as acetylcholine antagonists[333]. Doses of tremorine just sufficient to produce tremor and rigidity cause a rise of up to 50 per cent in the acetylcholine content of brain[334]. In a recent analysis[47], it has been shown that the differential action of tremorine on the so-called 'free' and 'bound' acetylcholine of brain is similar to that produced by eserine and other anticholinesterases and is similarly affected by atropine. Other drugs, such as anaesthetics and morphine, which also increase the acetylcholine content of brain, have different effects on its 'free' and 'bound' fractions. The actions of tremorine on the electroencephalogram also parallel those of eserine[331] and, although tremorine has negligible anticholinesterase activity[335], the liberation and accumulation of free acetylcholine would adequately explain its central pharmacological actions. It must, however, be recalled that some degree of anti-acetylcholine activity is commonly seen in drugs,

Table 5.7. Drugs used in the treatment of Parkinsonism

Approved Name	*Proprietary Name*	*Formula*	*Major Properties*
Benzhexol, Trihexyphenidyl	Artane, Pipanol		Atropine-like (mild CNS stimulant; peripheral parasympatholytic action)
Cycrimine	Pagitane		Similar to benzhexol
Biperiden	Akineton		Similar to benzhexol
Ethopropazine, Profenamine	Parsidol, Lysivane	see *Table 5.1.*	Peripheral parasympatholytic; central depressant.
Procyclidine	Kemadrin		Similar to ethopropazine
Benztropine	Cogentin	see *Table 5.2.*	Similar to ethopropazine but also antihistamine.
Orphenadrine	Disipal, Norflex	see *Table 5.2.*	Antihistamine; parasympatholytic; CNS stimulant.
Chlorphenoxamine	Phenoxane, Clorexan	see *Table 5.2.*	Similar to orphenadrine
Methixene	Tremonil		Blocks adrenaline β-receptors

such as the phenothiazines, that have Parkinsonian side effects. Moreover, methixene, a recent addition to the range of anti-Parkinsonian drugs, is said to be particularly effective against tremor yet its principal pharmacological action is to block adrenaline β-receptors.

It is not easy to formulate a testable hypothesis which will satisfactorily explain why a disease which is associated with a deficiency of brain dopamine should be best treated by agents which antagonize acetylcholine. Physiologically, the extrapyramidal symptoms seen in Parkinson's disease reflect an imbalance of excitatory and inhibitory influences in which the excitatory influence, though fluctuating, is dominant. If dopamine and acetylcholine were the central mediators of the inhibitory and excitatory influence respectively, a deficiency of dopamine would have the same effect as an excess of acetylcholine and the symptoms of Parkinson's disease might be relieved either by restoring dopamine to the brain or by inhibiting the otherwise unopposed action of acetylcholine. Although dopamine has inhibitory actions in some experimental situations (p. 271) and seems to be particularly associated with the extrapyramidal system, there is as yet no real evidence to sustain the hypothesis that Parkinson's disease is due to a removal of a dopamine mediated restraint on excitatory activity. There is, indeed, some evidence against it, since DOPA is not so effective an anti-Parkinsonian agent as are the acetylcholine antagonists: the tremor, in particular, is not improved and may be worsened by DOPA treatment[336]. The alternative possibility is that Parkinson's disease is primarily due to the liberation of free acetylcholine from the neurones of the basal ganglia which are as rich in acetylcholine as they are in dopamine. Over the past few years, neuropharmacological studies have focused predominantly to the detriment of acetylcholine on the more recently discovered neurohumoral factors and for this reason no information is available concerning the acetylcholine metabolism of brain samples taken from Parkinsonian patients. The possibility of a relationship between acetylcholine and dopamine metabolism also merits investigation. DOPA does not, however, reverse the effects of tremorine.

The symptoms of extrapyramidal involvement which accompany treatment by many of the psychotropic drugs are generally described as 'Parkinsonian'. Although the symptoms respond to treatment by anti-Parkinsonian drugs, they cannot be attributed either to dopamine deficiency or to acetylcholine excess. Thus, the extent of the spasticity produced in cats by chlorpromazine, prochlorperazine and mepazine bears no relation to the degree of dopamine depletion in the corpus striatum[337]. Neither chlorpromazine nor reserpine have a marked effect on the acetylcholine content of brain nor do they alter the proportion of 'free' ester[47].

In experimental animals, catatonia is the most obvious manifestation of the extrapyramidal stimulation which follows the administration of chlorpromazine or reserpine. A much more extreme form of this condition can be produced by bulbocapnine, but this drug, in doses sufficient to produce a maximal cataleptic effect, has no detectable action on either the acetylcholine or the dopamine content of brain[30]. The catalepsy due to bulbocapnine can however be partially antagonized by atropine and the intraventricular injection of acetylcholine produces a cataleptic-like condition in cats[80].

Finally, it should be recalled that DOPA can itself produce extrapyramidal effects as well as reversing them[140], that reserpine can reverse tremorine induced tremor in rats as well as producing Parkinsonian side effects in the human subject[339] and that, many years ago, bulbocapnine was used in the treatment of Parkinson's disease.

From the neuropharmacological aspect, therefore, it seems that the symptoms of Parkinson's disease itself may find an explanation more readily than the similar symptoms produced by psychotropic drugs. It is safe, however, to predict that the biochemical pharmacology of extrapyramidal disease will prove to be as perplexing as its pathology and as complex as the functional and structural interconnections of the many components of the basal ganglia.

Psychotomimetic Drugs

A number of psychotomimetic substances—including adrenochrome and adrenolutin which are derived from adrenaline—are indole compounds and are therefore structurally related to 5-hydroxytryptamine. The most extensively studied member of the group—LSD25—is, however, less closely related to 5-hydroxytryptamine than are compounds such as bufotenine.

In 1953, Gaddum demonstrated[3] that LSD antagonized the action of 5-hydroxytryptamine on a number of pharmacological preparations and he suggested that the central effects of LSD might be due to its exerting a similar antagonism at central synapses. This suggestion, with its implication that 5-hydroxytryptamine acted as a transmitter substance in the central nervous system was the immediate inspiration of a quite remarkable number of studies seeking to establish a relationship between 5-hydroxytryptamine activity and central nervous function. The later discovery of 5-hydroxytryptamine antagonists which have no psychotomimetic action (bromolysergic acid is the example most usually quoted) caused many to doubt that the actions of the psychotomimetic indole derivatives could be explained on the basis of their interaction with 5-hydroxytryptamine. The hypothesis cannot, however, be dismissed so easily, for substances that antagonize 5-hydroxytryptamine on smooth muscle preparations need not necessarily have the same action on the central nervous system. Thus, the sedative effect of 5-hydroxytryptamine, injected into the cerebral ventricles, is antagonized by LSD but not by bromo-LSD[339] and there is evidence that the latter compound may not reach LSD sensitive areas as easily as does lysergic acid itself[340]. Moreover, although some 5-hydroxytryptamine antagonists are not psychotomimetic, all the psychotomimetic indoles certainly antagonize 5-hydroxytryptamine.

It is important to recognize that, even on such simple pharmacological preparations as the uterus, lysergic acid is not invariably antagonistic to 5-hydroxytryptamine, facilitation being observed with lower doses[341]. On some preparations (including dog blood pressure and intestinal motility), only facilitation has been reported[342,343] and on others no agreement has been reached concerning the interaction between 5-hydroxytryptamine and lysergic acid. Thus, the literature contains a report that 5-HTP antagonizes[344], and another that it has no effect on[345], the psychotomimetic action of lysergic acid. The reported behavioural effects of LSD in animals vary

between sedation and sham rage; LSD has been said both to inhibit[346] and to potentiate[347] the 5-hydroxytryptamine induced prolongation of barbiturate-narcosis. On the EEG, LSD and 5-hydroxytryptamine (or 5-HTP) have similar effects in some areas of the brain and opposite effects in others[348]. Both compounds inhibit trans-callosally evoked potentials in the visual cortex and chlorpromazine is equally antagonistic to both[81,349].

While disparate results such as these may be partly explained by the fact that the mutual antagonism and synergism of LSD and 5-hydroxytryptamine are dose dependent, the results themselves do not permit the drawing of firm conclusions concerning the actual mode of action of either compound. Thus, although there is certainly suggestive evidence that the psychotomimetic actions of LSD depend on its relationship with 5-hydroxytryptamine, the nature of the relationship is far from clear and it is likely to remain so until more is known of the physiological role of 5-hydroxytryptamine.

The other psychotomimetic compounds which contain an indole nucleus have been much less extensively studied than LSD. Bufotenine is found in the toad, *Bufo marinus*, in a toadstool and in cohaba snuff; both bufotenine and *NN*-dimethyltryptamine (which also occurs in cohaba snuff) can be formed *in vitro* from 5-hydroxytryptamine and there have been reports that the urine of schizophrenic patients contains bufotenine[350]. This finding could not be confirmed in a later study[351] and it seems unlikely that bufotenine production is an aetiological factor in schizophrenia.

Attempts to explain the hallucinogenic action of LSD on biochemical grounds have proved unsuccessful. Those biochemical actions which have been reported and which include inhibition of glucose oxidation[352,353], inhibition of pseudocholinesterase[345] and elevation of the ATP:ADP ratio in brain[355], are non-specific or require large doses of the drug for their demonstration.

Mescaline is structurally similar to dopamine but some of its biochemical and physiological actions are similar to those of LSD. In particular, both compounds inhibit glycolysis in electrically stimulated brain slices *in vitro*[356,357] and inhibit trans-callosally evoked potentials *in vivo*[358]. The psychogenic actions of mescaline do not occur coincidentally with the time of maximum blood levels[359] and it has been suggested that they are due to a mescaline metabolite such as 2-(3,4,5-trimethoxyphenyl) ethanol (*Table 5.6*) which itself has hallucinogenic activity, particularly in individuals subjected to stroboscopic stimulation[360]. Mescaline has sympathomimetic effects which develop concurrently with the hallucinatory state and it is interesting that other sympathomimetic compounds such as amphetamine, which are not usually regarded as hallucinogens, do have psychogenic activity in some subjects.

A common mechanism may underlie the psychogenic action of the indole type hallucinogens on the one hand and the compounds related to adrenaline on the other, but it is not yet possible to say whether this action is related to 5-hydroxytryptamine or to adrenaline. Adrenochrome and adrenolutin have structural similarities with the indoles and, under similar conditions, both LSD and mescaline similarly antagonize the action of 5-hydroxytryptamine on isolated preparations[341]. There is some evidence that LSD

antagonizes the action of adrenaline as it does that of 5-hydroxytryptamine[361] and both LSD and mescaline have been reported to increase the conversion of adrenaline to adrenochrome and adrenolutin *in vivo*[362]. The mode of action of these compounds themselves is not known, though they inhibit glutamic decarboxylase[363] and this may contribute to their hallucinatory activity in view of the excitatory actions of glutamic acid.

The relationship of many of the psychotomimetic compounds to naturally occurring substances (*Table 5.6*) has led several groups of workers to suggest that disturbances of monoamine metabolism in man may produce hallucinogenic metabolites and thus be an important aetiological factor in schizophrenia. Among the hallucinogens which have been reported or sought for in the blood and urine of schizophrenic patients are adrenochrome, adrenolutin, 3,4-dimethoxyphenylethylamine, bufotenine and tryptophan and its metabolites[361,364,365,367]. Although considerable controversy surrounds the whole question of the actual occurrence of these compounds *in vivo* and of their particular relationship to the schizophrenic process, sufficiently persuasive evidence has been forthcoming to suggest that further investigation of the relationship between monoamine metabolism and schizophrenia will be worth while. It has recently been reported[366] that melanin production is increased in schizophrenic subjects and it has been cautiously suggested that this may result from a lack of the 'lightening factor', melatonin, which is itself produced from 5-hydroxytryptamine.

N-Methyl-3-piperidyl benzilate (*Table 5.6*) has anti-acetylcholine activity and some other compounds such as atropine with a similar pharmacological action are psychogenic in some individuals. Phencyclidine is an anaesthetic which particularly blocks impulse transmission from the thalamus to the cerebral cortex. The fact that it has psychotomimetic activity is interesting in view of the observation, already mentioned, that perceptual deprivation produces hallucinations in man. Since cholinergic synapses are widely distributed in the brain it is possible that the psychogenic action of anti-acetylcholine compounds is also related in some way to an interference with sensory input to the brain.

The relationship between the hallucinogenic activity of compounds or experimental procedures which cause perceptual deprivation and those essentially stimulant compounds whose activity seems to stem from their interaction with monoamine metabolism cannot yet be established nor is it possible to specify the actual locus of action of the psychotomimetic drugs.

Extensive reviews dealing with the pharmacology and biochemistry of the psychotomimetic compounds[367,368] and with biochemical theories of schizophrenia[369,370], have recently been published.

CONCLUSIONS

The accidental discovery of psychotropic properties among a number of different compounds has been exploited by chemists to produce a huge range of psychotropic drugs, a few of which have been discussed in this review. While there can be no doubt that some of them have been of the greatest therapeutic value—the treatment of schizophrenia, for instance, has been revolutionized by the phenothiazine compounds—others have failed to

justify the enthusiasms aroused by the early results of their clinical application. It is also unfortunately true that a rational explanation of their mode of action cannot yet be presented.

So far as the clinical aspects of psychotropic drug action are concerned, the greatest obstacle to a realistic assessment of the worth of individual compounds is undoubtedly the existence of the placebo effect which is not necessarily eliminated even in the most carefully designed double-blind trials. Many of the psychotropic drugs have pronounced peripheral effects which make it impossible to disguise from both the patient and his attendants, the fact that a drug has been administered. One method of circumventing this difficulty is to produce dummy mixtures which have no central action but do have peripheral actions similar to that of the drug under trial. Some trials have been made with 'active dummies' but many of the substances which might be used to produce the required peripheral effects have inevitably a central action also. Furthermore, part of the therapeutic effect of the psychotropic drugs may itself be attributable to peripheral activity. While this may not detract from their clinical value, it produces a perplexing situation for the experimental neuropharmacologist investigating the central actions of drugs in the hope of detecting the biochemical changes underlying the conditions for which they are prescribed. A direct pharmacological analysis of the clinical condition is precluded by the impossibility of reproducing the state in experimental animals. Thus, while it is possible to induce diabetes mellitus in some animals by removal of the pancreas, neurotic and psychotic conditions can at best only be caricatured, and that with difficulty, by experimental methods.

An explanation of the mode of action of psychotropic drugs in terms of their effect on neurohumoral systems demands broadly based investigations into the actions of a few representative drugs. While many studies are concerned with only one neurohumoral system, the evidence suggests that many of the more successful therapeutic agents interact with more than one neurohumoral factor. Nothwithstanding the large number of drugs involved, structure–activity relationships among the psychotropic compounds have not revealed, except in a very few instances, any clear indication of the nature of the receptors and the chemical mediators with which the drugs interact.

Experiments in which psychotropic drugs are used in attempts to elucidate the function of neurohumoral substances have to be interpreted cautiously. A compound may antagonize the action of a neurohumour on a simple pharmacological preparation but it is an almost inevitable consequence of the fact of this antagonism that the same compound, under different conditions or on different preparations, will also mimic the neurohumour or, by preventing its destruction, may prolong its action. In addition, it is likely to have its own independent properties, so that its action on the complex receptor system of the brain cannot be adequately determined by analysing the rather simple and frequently imprecisely defined behavioural data provided by many types of pharmacological study. When the investigation involves the interaction of two drugs, the problem becomes more than twice as complicated. The presence of co-ordinated and reciprocally related systems of inhibitory and excitatory nuclei in the central nervous system, together with the fact that drugs may have excitatory or inhibitory effects

depending on their concentration, adds a final complication to an already involved situation. For this reason, as has been emphasized throughout this review, it seems that a knowledge of the physiological significance of neurohumoral substances is more likely to come from studies of their direct actions on synaptic and neuronal processes rather than from considerations of the gross behavioural effects produced by compounds which appear to have some structural or functional relationship with the neurohumours.

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6

THE NITROFURANS

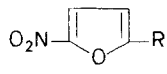
K. MIURA AND H. K. RECKENDORF

INTRODUCTION TO HISTORICAL DEVELOPMENT

CHEMOTHERAPEUTIC agents are defined as chemical compounds which affect causative organisms without injuring the patient. The nitrofurans, which do not occur in nature and belong to this large group of compounds, are characterized by a heterocyclic ring consisting of four carbon atoms and one of oxygen; an azomethine group, —CH=N—, is attached at C_2 and a nitro group at C_5. Antimicrobial effectiveness of all furfural derivatives requires two essential factors:

(1) the presence of a nitro group attached to C_5 of the furan ring;
(2) one of the many variations of the side chain at C_2.

The simplest representation of these nitrofurans is therefore as follows:



which, for convenience, is shown in this review as 5-O_2N·Fur·R. The prime reasons for the growing interest in these compounds for the treatment of infectious diseases are that resistant strains have not developed with their use, and that they exhibit specific action in eradicating certain types of bacteria. Moreover, nitrofurans have shown minimal toxicity and side effects during prolonged use. They are also widely employed in the treatment of animal disease and in promoting growth and feed utilization in farm animals.

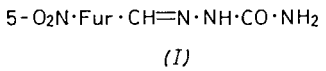
Although some characteristics of the nitrofurans, for example, their mechanism of action, remain incompletely explained, the physician is nevertheless able to use the drugs for some specific indications. Perhaps this explains why so few resistant strains have developed with their use in comparison to other chemotherapeutic agents, notably the antibiotics.

The search for more effective and less toxic nitrofuran derivatives continues to yield better compounds. Some of the recently discovered products have aroused keen interest in researchers and it is hoped that new compounds of the nitrofuran group can soon be used in medical practice in lieu of the present less specific administration of broad spectrum antibiotics. The interesting history of nitrofurans is treated in detail by Dunlop and Peters*.

Nitration of furfural was successfully carried out in 1930, almost 100 years after its discovery. Several nitrated compounds which were studied in following years had to be abandoned because of dangerous side effects. In 1939 a programme of research with new nitrofurans was initiated by Eaton

* *The Furans*, Reinhold Publishing Corporation, New York, 1953.

Laboratories (U.S.A.) and led to the discovery of nitrofurazone by Dodd and Stillman[1] in 1944. This important product can be called one of the fundamental discoveries in the history of nitrofurans; it was successfully used in combating serious wound infections on the European continent during World War II. Although it is still available as a topical agent, it lacks effectiveness against certain organisms, such as those occurring in burns,



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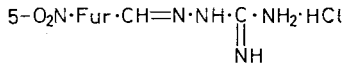

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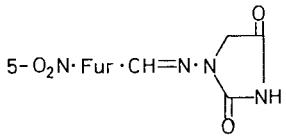

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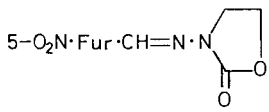

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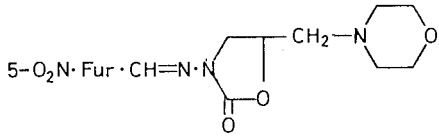

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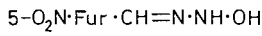

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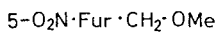

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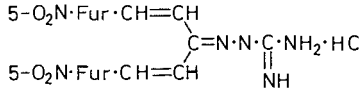

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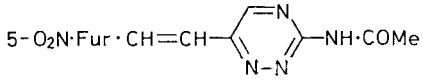

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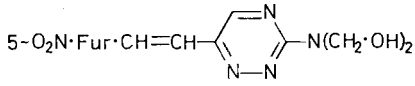


(X)

and thus the search for new derivatives continues. So far, however, a superior product has not been marketed.

A few years after the introduction of nitrofurazone, two additional nitrofurans were selected from several hundred synthesized compounds for use in human and veterinary medicine. These two products were nitrofurantoin and nitrofurazolidone which are used in urinary tract and intestinal infections, respectively. It has been difficult to surpass nitrofurazone for topical use, but it seems even more difficult to improve upon these two latter products. Numerous attempts have either been made or are in process; it will be a part of the following description to mention some of the most recent discoveries, none of which have become available for general use.

In order to fill the gap in the specific treatment of systemic staphylococcus diseases, the compound furaltadone was introduced in the U.S.A. and a few European countries, but had to be withdrawn later because of its neurotoxic side effects. This product is, however, still available for veterinary use.

In addition to the above-mentioned nitrofurans, Furaspor was employed

clinically for the treatment of microsporum infections, especially *Tinea capitis*. The same product later proved to be effective in dermatomycosis. For limited use in veterinary medicine the product Furamazone was recently marketed because of its favourable effects in bovine intestinal infections. One of the most recently introduced nitrofurans is Mycofur which is used for the treatment of fungal and bacterial infections, and especially when combined with furazolidone for topical use in the treatment of moniliasis and trichomonas infections. The introduction of oral agents, however, for the treatment of trichomonas infections has rendered the furazolidone-Mycofur combination obsolete, but it would be premature to appraise the long-term results of oral treatment at the present time.

In view of thousands of publications that have appeared during recent years, it is difficult to summarize all aspects of the chemistry and clinical uses of nitrofurans, not to mention the wide range of experimental studies which were their necessary predecessors. In the clinical section of this chapter each drug will be described under a separate heading with emphasis on the progress in human medicine. The reader should understand in addition that the nitrofurans also have a definite place in veterinary medicine. Besides some basic work which was published soon after the compound under discussion appeared on the market, only the most recent publications have been considered. Hence, the list of references is not to be considered complete.

CHEMICAL AND PHYSICAL PROPERTIES OF NITROFURAN COMPOUNDS

Amongst forty-two furan derivatives which were investigated by Dodd and Stillman[1] in 1944 nitrofurazone was found to be highly effective as a topical agent. It contains the azomethine side chain (—CH=N—) in the 2-position of the furan ring. From 1944 to 1960 over 450 similar compounds were synthesized and studied for antimicrobial properties. Seven compounds were selected from these, namely the commercially available compounds nitrofurazone, nifuroxime, guanofuracin hydrochloride, nitrofurantoin, furazolidone and panazone. Recently acetyl- and di-(hydroxymethyl)-derivatives of panfuran have been developed for clinical use in Japan. Some physical and chemical properties of these furan derivatives are shown in *Table 6.1.*

The clinically used nitrofuran compounds may be divided into four groups: (1) azomethine type nitrofurans; (2) di-(nitrofuran) compounds; (3) vinylogous nitrofurans; (4) other nitrofurans.

Azomethine Type Compounds

A few nitrofuran azomethine compounds are soluble in water. But guanofuracin and furaltadone are soluble only in acidic solution by salt formation. The free base precipitates rapidly by addition of alkali to the solution. It is known that the water solubility of nifuroxime and nitrofurantoin increases by adding alkali, and a sodium salt of nitrofurantoin can be prepared for practical purposes. Most of the nitrofurans are readily soluble in polyethyleneglycols and in dimethylformamide. A solution of the desired concentration of nitrofurans can sometimes be prepared by dissolving the compound

Table 6.1. Physicochemical properties of clinically effective nitrofurans[2–5]

Compound number	*M.wt.*	*Appearance*	m.p. (*decompn.*) (°C)	*Ultra-violet absorbency maxima* (mμ)	*Approx. solubility* (mg/l)			
					Water	*Ethanol 95%*	*Peanut oil 30°C*	*Chloroform*
I	198·14	Pale yellow needles	227–241	260·0, 375·0	210	920	15	22
II	233·63	Yellow crystal powder	254	255·0, 364·0	10,000	1,670	100	100
III	238·16	Orange yellow needles	270–272	265·0, 367·5	190 (pH7)	510	21	35
IV	225·16	Yellow crystals	255	259·0, 367·0	40	90	10	200
V	304·29	Yellow crystals	206	275·5, 365·0	750	1,320	20	22,000
VI	156·10	Orange yellow prisms	163–164	232·5, 341·0	1,040	39,600	3,000	5,400
		Pale yellow needles	129–130	232·5, 348·0	4,450	11,000		
VII	157·12	Light yellow viscous oil	6·5–7·25	230·0, 319·0	11,000	–	170,000	Completely miscible
VIII	396·76	Orange red powder	300	320·0, 417·5	10	100	50	25
IX	275·22	Pale yellow	275	286·0, 382·0	10	20	10	16·6
X	293·25	Orange red crystals	153–161	292·0, 405·0	5	62·5	15	33·3

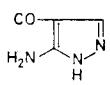
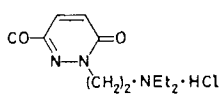
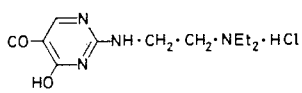
Key to compounds (see p. 321 for formulae):

I 5-Nitro-2-furfuraldehyde semicarbazone (nitrofurazone, nitrofural, NF-7, Furacin, Sanfuran).
II 5-Nitro-2-furfurylideneaminoguanidine hydrochloride (guanofuracin hydrochloride); (NF-56 is the sulphate).
III 1-(5-Nitro-2-furfurylideneamino)hydantoin (nitrofurantoin, NF-153, Furadantin, Furantoin).
IV 3-(5-Nitro-2-furfurylideneamino)oxazolidin-2-one (furazolidone, NF-180, Furoxone, Medaron, Puradin, Trifurox).
V 5-(Morpholinomethyl)-3-(5-nitro-2-furfurylideneamino)oxazolidin-2-one (furaltadone).
VI 5-Nitro-2-furfuraldehyde oxime (nifuroxime, nitrofuroxime, NF-6, Micofur).
VII 5-Nitro-2-furfuryl methyl ether (furaspor).
VIII 1,5-Bis-(5-nitro-2-furyl)penta-1,4-dien-3-one guanylhydrazone hydrochloride (panazone).
IX 3-Acetamido-6-[2-(5-nitro-2-furyl)vinyl]-1,2,4-triazine (acetylpanfuran).
X 3-Di-(hydroxymethyl)amino-6-[2-(5-nitro-2-furyl)vinyl]-1,2,4-triazine (Panfuran S).

in a small volume of the organic solvent and introducing this solution into water.

The aqueous solution of nitrofurans is not stable in alkali and many nitrofurans are relatively insoluble in water. Many attempts have been made to find water-soluble and active nitrofurans. It was reported[6] that guanofuracin lactate and gluconate are more readily soluble than guanofuracin hydrochloride in water. However, antibacterial activity of these organic acid salts is identical to that of hydrochloride[3].

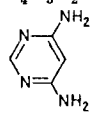
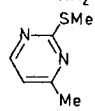
Table 6.2. *N*-Acyl derivatives of 5-nitrofurfural hydrazone
$5\text{-}O_2N\cdot Fur\cdot CH{=}N\cdot NHR$

Compound number	R	m.p. (°C)	*References*
1	$CO\cdot NH_2$ (nitrofurazone)	224–6	7–13
2	$CO\cdot NHMe$ (NF-62)		
3	$CO\cdot NH\cdot CH_2\cdot CH_2\cdot OH$ (NF-145)		14
4	$CO\cdot NH\cdot NH_2$	203–4·5	15, 16
5	$CO\cdot NH\cdot N{:}CH\cdot Fur\cdot 5\text{-}NO_2$	260·5–1·5	16
6	$CO\cdot CO\cdot NH_2$ (nifuraldezone)		14
7	$CS\cdot NH\cdot CH_2Ph$	148–9·5	17
8	COMe (nihydrazone)	230–5	18
9	$CO\cdot CH_2Cl$	245–6·5	19
10	$CO\cdot CHCl_2$	203–5	20
11	$CO\cdot CH_2\cdot CN$	190–92d.	19
12	$CO\cdot (CH_2)_3\cdot OH$	143	21
13	$CO\cdot C_6H_4\cdot OH(o)$	276–7d.	22
14	$CO\cdot C_6H_4\cdot OH(p)$	298	25
15		219	23
16	$CO\cdot C_5H_4N$[a]	258–60d.	24, 25
17		240d.	26
18		246d.	27
19		197–9	27
20	$CO\cdot OEt$	195–6	28
21	$CO\cdot O\cdot CH_2\cdot CH_2\cdot OH$	168	28
22	$P(OEt)_2$ ‖ S	101·5–2·5	29

[a] 4-pyridyl

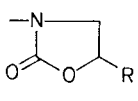
The substituents R and R′ which have been introduced into the azomethine group may be classified as acyl (*Table 6.2*), alkyl (*Table 6.3*) and a mixture of both of these types (*Table 6.4*).

Table 6.3. N-Alkyl derivatives of 5-nitrofurfural hydrazone
$5\text{-}O_2N\cdot Fur\cdot CH{=}N\cdot NRR^1$

Compound number	R	R^1	m.p. (°C)	*References*
1	H	Et	119–21	30
2	H	$CH\cdot(OH)Cl_2$	245	31
3	H	$CH_2\cdot CO\cdot NH_2$	167–70	32
4	H	$C_6H_4\cdot SO_2\cdot NH_2$ (*p*)	220–30	39
5	H	$CH_2\cdot CH_2\cdot C_5H_4N$[a]	148·5–51	34
6	H	$C_4H_3N_2$[b]	228–9d.	35
7	H		272–5d.	36
8	H		250–2d.	35
9	Me	$CH_2\cdot CO\cdot NH_2$	181·5–4	32
10	Me	$CH\cdot(OMe)Ph$	107·5–8·5	37
11	Me	$CH\cdot(OMe)\cdot Fur\cdot 5\text{-}NO_2$	130–1	37

[a] 2-pyridyl [b] 2-pyrimidyl

A large number of azomethine nitrofurans containing various heterocyclic rings have been synthesized: the most important of these contain either a hydantoin (*Table 6.5*) or oxazolidinone (*Table 6.6*) ring. Nitrofurans containing the following rings have also been studied: 3-oxazolidin-2-one (*XI*)[43,44], 3-rhodanine (*XII*)[45], 3-imidazolid-2-one (*XIII*)[46], 1-hydantoin (*XIV*)[47,47a], 1-imidazolidine-2-thione (*XV*)[48], 4-1,2,4-triazole (*XVI*)[49], 1-piperazine (*XVII*)[50], 4-tetrahydro-1,4-thiazine-1,1-dioxide (*XVIII*)[51], 1-tetrahydropyrimidin-2-one (*XIX*)[52], 5-methyl-4,5-dihydrouracil (*XX*)[53].



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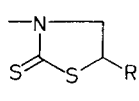

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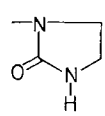

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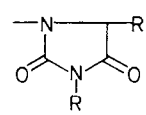

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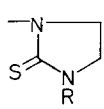

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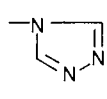

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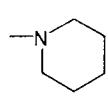

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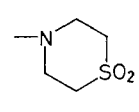

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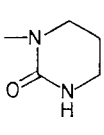

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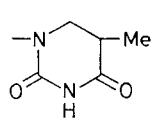


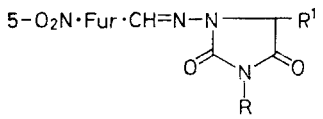
(XX)

Table 6.4. *N*-Acyl-*N*-alkyl derivatives of 5-nitrofurfural hydrazone
5-O_2N·Fur·CH=N·NRR^1

Compound number	R	R^1	m.p. (°C)	*References*
1	CO·NH_2 (nidroxyzone)	CH_2·CH_2·OH	214–6d.	14
2	CO·NH_2	CH_2·CH_2·COMe	177–8	38
3	CO·NH_2	CH_2·CH_2·C_3H_4N[a]	235	30
4	CO·NH_2	CH_2·CH_2·C_4H_8NO[b] HCl	250–1	34
5	CO·NH_2	Bu	144–6	28, 39
6	CO·OEt	Me	145–6	28
7	CO·OEt	CH_2·CH_2·OH	143–7	28
8	COMe	CH_2·CH_2·CN	188–90	40
9	COMe	CH_2·CH_2Cl	145·5–6·5	41
10	COMe	CH_2·CO·NH_2	—	42

[a] 2-pyridyl [b] morpholino

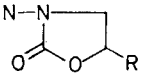
Table 6.5. Aminohydantoin derivatives of 5-nitrofurfural

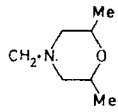
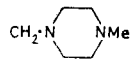
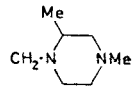
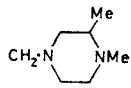
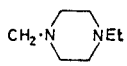
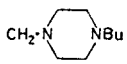
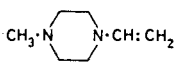


Compound number	R	R^1	m.p. (°C)	*References*
1	H (nitrofurantoin)	H	261, 272	47–59a
2	H	CH_2·OH	205–7	60, 61
3	H	$CH_2(CH_2)_4Me$	146–6·5	62
4	H	CH_2·$(CH_2)_5Me$	137–8	62
5	H	CH_2·$(CH_2)_6Me$	122–3	62
6	H	CH_2·$(CH_2)_8Me$	120–2	62
7	H	CH_2Ph	156–7	62
8	H	CH_2·O_2C·C_6H_4·COOH (*o*)	136–8	63
9	COMe	H	200–4d.	64
10	CH_2OH	H	—	64
11	CH_2·CH_3·OH	H	236–8d.	64
12	CH_2·CH_2·CN	H	195–200	65
13	CH_2·CMe_3	H	195–9	66
14	CH_2·CH_2·NMe_2	H	227–30d.	64
15	CH_2·$(CH_2)_2$·NMe_2	H	238–41d.	64
16	CH_2·$(CH_2)_3$·NMe_2	H	223–5	64
17	CH_2·$(CH_2)_2$·NEt_2	H	238–9d.	64
18	CH_2·CH_2·NPr^i_2	H	230–40d.	64
19	CH_2·$(CH_2)_2$·NPr^i_2	H	224–7d.	64
20	CH_2·C_6H_4·NO_2·(*p*)	H	218–21d.	66
21	CH_2·C_5H_4N[a]	H	210–2d.	64
22	CH_2·$(CH_2)_3$·C_5H_4N[a]	H	229–33d.	64
23	CH_2·CH_2·C_4H_8NO[b]	H	225	64
24	CH:CH_2	H	170–2	67
25	CH:CHMe	H	167–8	67
26	CH_2·CH:CHMe	H	170–2	67
27	CH:CHMe	H	237–40	67
28	CO·OMe	H	224–7	66
29	CO·OEt	H	218–20	66
30	CO·OPr^i	H	205–7	66
31	CO·OPr^n	H	194–7	66
32	CO·OBu	H	189–91	66
33	CO·O·C_5H_{11}	H	176–7	66
34	CO·O·C_6H_{13}	H	170–2	66
35	CO·O·CH_2Ph	H	195–8	66

[a] 4-pyridyl [b] morpholino

Table 6.6. Amino-oxazolidin-2-one derivatives of 5-nitrofurfural



Compound number	R	m.p. (°C)	*References*
1	H (furazolidone)	225d.	59, 68–79
2	$CH_2 \cdot NEt_2$	170–1	80
3	$CH_2 \cdot NBu_2$	152·5–3·5	80
4	$CH_2 \cdot NPhMe$	214–15	80
5	$CH_2 \cdot NPh_2$	163–5	80
6	$CH_2 \cdot C_4H_8N$[a]	207–8	80
7	$CH_2 \cdot C_5H_{10}N$[b]	197–8	80
8	$CH_2 \cdot C_4H_8ON$[c] (furaltadone)	205–6	80–84
9	$CH_2 \cdot N \cdot (CH_2 \cdot CH{:}CH_2)_2$	151	84
10		203	84
11		197	84
12		191–3	84
13		161–4	84
14		193	84
15		186	84
16		184–5	84

[a] pyrrolidino [b] piperidino [c] morpholino

Di-(nitrofuran) Type Compounds

$(5\text{-}O_2N \cdot Fur \cdot CH{=}CH)_2C{=}N \cdot N \cdot RR'$

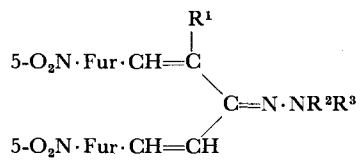
Panazone (*Tables 6.1 and 6.8*) which was synthesized[3] in 1951, is a typical di-(nitrofuran) compound, being insoluble in water and unstable to heat. When panazone is heated in organic solvents, the solution changes colour[85] (*Table 6.7*).

Table 6.7. The effect of heating on the antibacterial activity of panazone[85] and the subsequent changes of colour of the solution

Time of heating at 120° C (min)	*Change in colour of solution by heating*	*Concentration which carries a bacteriostatic effect on the dysentery bacillus*
0	scarlet red	1: 20,000
5	orange red	1:1,280,000
10	orange	1:2,560,000
30	yellow	1:5,120,000
60	soiled yellow	1:2,560,000
120	dark yellow	1: 640,000
5 (at 200°)	yellow	1:5,120,000

For example, the original scarlet red colour changes to yellow on heating for 30 minutes at 120° and the chemical structure of the compound changes considerably (see p. 338). The bacteriostatic activity of the yellow solution against dysentery bacillus is about 250 times greater than that of panazone. This observation has prompted the synthesis of other di-(nitrofuran) derivatives, some of which are listed in *Table 6.8.*

Table 6.8. Di-(nitrofuran) azomethine type compounds



Compound number	R^1	R^2	R^3	m.p. (°C)	*References*
1	H (panazone)	H	C·NH₂·HCl ‖ NH	196–7d.	3, 4
2	H	H	C·NHMe ‖ NH	242d.	86
3	H	H	C·NMe₂ ‖ NH	163d.	86
4	H	H	C·NHEt ‖ NH	169–171d.	86
5	H	H	C·NHPr ‖ NH	173–175d.	86
6	Me	H	C·NH₂ ‖ NH	272d.	87
7	H	COMe	C·NH·COMe ‖ NH	219–20d.	88
8	H	H	CO·NH₂	233d.	85
9	H	H	CS·NH₂	205d.	85
10	H	H	Ph	125d.	85

Vinylogous Nitrofurans

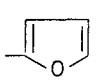
The azomethine group, —CH=N—, in these compounds is separated from the furan ring by an additional vinyl group, —CH=CH—. Panfuran (*Table 6.9*) is a typical example; the base is insoluble in water but soluble in acidic solution; the hydrochloride is soluble to the extent of one per cent. The important compounds of the panfuran series for medicinal use are the acetyl- and di-(hydroxymethyl) derivatives, neither of which is readily soluble in water. The former is a stable compound whereas the latter is unstable.

Physical, chemical and thermal treatment will cause di-(hydroxymethyl)-panfuran (Panfuran S) to decompose to panfuran base and to formaldehyde. The same result is obtained by heating a mixture of di-(hydroxymethyl)-panfuran and a 10 per cent sulphuric acid solution[5]. The metabolic degradation of di-(hydroxymethyl)panfuran *in vivo*, however, is still not fully understood.

Nitrofurans containing up to four vinyl groups have also been studied[88a]. These were prepared from the corresponding aldehyde and a compound containing a primary amino group:

$$5\text{-}O_2N\cdot Fur\cdot(CH{=}CH)_n\cdot CHO + RNH_2 \rightarrow 5\text{-}O_2N\cdot Fur\cdot(CH{=}CH)_n\cdot CH{=}NR$$

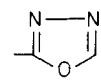
Among the most active compounds were those in which R was –OH, $-NH\cdot CS\cdot NH_2$ or $-NH\cdot C{=}NH\cdot NH_2$ and *n* was 2 or 3.



(XXI)

(XXII)

(XXIII)



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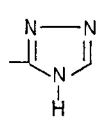

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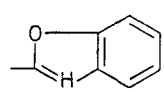
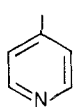


(XXVII)

(XXVIII)



(XXIX)

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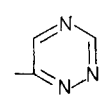
(XXXI)



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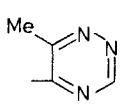

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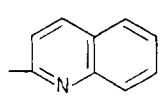

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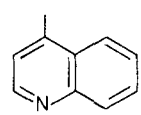

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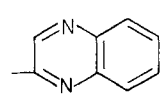

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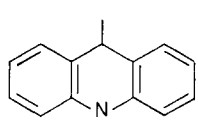


(XXXVII)

Since 1961, a large number of heterocyclic vinylogues of the simpler nitrofurans have been synthesized by using various heterocyclic nuclei instead of the aminotriazine ring of panfuran. With these modifications nitrofuran vinylogues now total more than 500. Tests for antimicrobial activity show that compounds containing the following heterocyclic rings possess the highest activity:

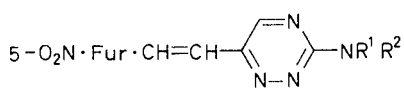
$$5\text{-}O_2N\cdot Fur\cdot CH{=}CH\cdot R \quad (R\text{: heterocyclic ring.})$$

2-furan (*XXI*)[89], 2-thiophen (*XXII*)[90], 2-thiazole (*XXIII*)[91], 2-(1,3,4-oxadiazole) (*XXIV*)[92], 2-(1,3,4-thiadiazole) (*XXV*)[91], 2-(1,2,4-triazole) (*XXVI*)[93], 2-benzoxazole (*XXVII*)[94], 2-pyridine (*XXVIII*)[91], 4-pyridine (*XXIX*)[91], 3-pyridazine (*XXX*)[95], 4-pyrimidine (*XXXI*)[91,96], 6-(1,2,4-triazine) (*XXXII*)[97], 5-(6-methyl-1,3,4-triazine) (*XXXIII*)[93], 2-quinoline (*XXXIV*)[91,98] 4-quinoline (*XXXV*)[91], 2-quinoxaline (*XXXVI*)[91] and 9-acridine (*XXXVII*)[91].

Compounds in which one of the ethylenic hydrogen atoms has been replaced by a 2-furyl group represent an interesting variation; furthermore, these compounds do not contain the usual —C=N— grouping but instead they have an amide group. These water-insoluble compounds are used in Japan in dermatology, veterinary medicine and as animal-feed additives. *Trans*-3-(5-nitrofuryl)-2-(2-furyl)acrylamide is an example[98a].

The important nitrofuran vinylogues are listed in *Tables 6.9–6.14*.

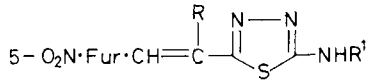
Table 6.9. 5-Nitrofurylvinyltriazine derivatives



Compound number	R^1	R^2	m.p. (°C)	*References*
1	H (panfuran)	H	270d.	4, 5
		HCl	300	99, 100
		HBr	200	
2	H (acetylpanfuran)	COMe	275, 278	97, 99, 100
3	COMe	COMe	204	92
4	H	COEt	261–2d.	101
5	H	$COBu^t$	210–211	101
6	H	$CO\cdot C_9H_{19}$	198–200	101
7	H	$CO\cdot C_{15}H_{31}$	168–9	101
8	H	$CO\cdot CH_2Cl$	295d.	101
9	H	COPh	252–4	92
10	H	Me	235	86
11	H	Et	187–8	86
12	H	Pr	194–5	86
13	Me	Me	210–11	86
14	H	$CH_2\cdot NMe_2$	136	5
15	H	$CH_2\cdot C_5H_{10}N$[a]	201	5
16	H	$CH_2\cdot C_4H_8NO$[b]	189	5
17	CH_2OH (panfuran S)	$CH_2\cdot OH$	158–61d.	5
18	H	$:CH_3$	268d.	5

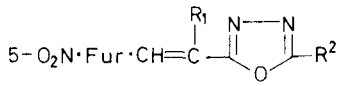
[a] piperidino [b] morpholino

Table 6.10. 5-Nitrofurylvinylthiadiazole derivatives[91a,102]



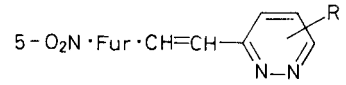
Compound number	R	R^1	m.p. (°C)
1	H	H	245d.
2	H	COMe	300
3	H	$CO \cdot CH_2Cl$	202d.
4	Br	H	228d.
5	Br	COMe	276–7
6	Me	H	230
7	Me	COMe	283
8	Et	H	202
9	Et	COMe	272d.
10	H	$CH_2 \cdot SO_3Na$	255–7d.
11	H	CO·NHMe	267–8d.

Table 6.11. 5-Nitrofurylvinyloxadiazole derivatives[13, 92, 103–5]



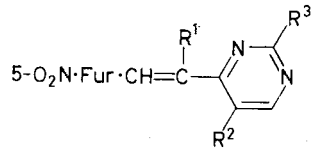
Compound number	R^1	R^2	m.p. (°C)
1	H	OH	214
2	Me	OH	206–7
3	Et	OH	171–2
4	Ph	OH	96–7
5	Et	SH	107
6	H	SMe	238d.
7	Me	SMe	176
8	Et	SEt	105
9	H	H	174–5
10	Me	H	157–8
11	Et	H	125–5·5
12	H	Me	226
13	Me	Me	154–6
14	Et	Me	107
15	H	CH_2Cl	161
16	Me	CH_2Cl	112–3
17	H	Ph	207–8
18	Me	Ph	190
19	Et	Ph	177–8
20	H	NH_2	272d.
21	Et	NH_2	230–2d.
22	Ph	NH_2	192
23	H	NHMe	218–9
24	H	NHEt	229
25	H	NHPr	181–2·5
26	H	NHBu	139
27	NHPh	NHPh	243
28	Me	NHMe	225
29	Me	NHEt	208–8·5
30	Me	NHBu	198
31	Me	NHPh	278
32	Et	NHMe	188–9
33	Et	NHEt	192
34	Et	NHBu	158–60
35	Ph	NHMe	124–6
36	Ph	NHEt	198–201
37	Ph	NHPh	221–2
38	Ph	NHBu	155–8
39	Me	NH_2	286d.

Table 6.12. 5-Nitrofurylvinylpyridazine derivatives[98]



Compound number	R	m.p. (°C)
1	4-NHCOMe	292
2	6-NH_2, HCl	290
3	4-OMe	202–3
	(*N*-oxide)	(244–5)
4	4-SMe	208–9
5	4-SO_2Me	218–9
6	4-NH·CH_2·CH_2·OH	255d.
7	4-NH·CH(CH_2·OH)$_2$	170–2
8	4-O(CHOH)$_2$Me	147–8d.

Table 6.13. 5-Nitrofurylvinylpyrimidine derivatives[105–7]



Compound number	R^1	R^2	R^3	m.p. (°C)
1	H	CO·OEt	NH_2	237
2	Me	H	NH_2	218–8·5
3	Et	H	NH_2	222
4	Pr^i	H	NH_2	152–5
5	H	H	NHMe	195–6
6	H	H	NMe_2	168–71
7	H	H	NH·C_5H_{11}	130–3
8	H	H	C_4H_8NO[a]	192–4
9	H	H	$C_5H_{10}N$[b]	129–31
10	H	H	Me	171
11	H	CO·OEt	Me	221–5

[a] morpholino [b] piperidino

Other Types of Nitrofurans

1. *5-Nitrofurylbutadienyl heterocyclic compounds*

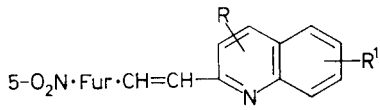
The butadienyl derivatives, which are similar to panfuran, have been investigated and have the following formula:

$$5\text{-}O_2N\cdot Fur\cdot CH{=}CH\cdot CH{=}CHR$$

in which R is 2-(1,3,4-oxadiazolyl) (*XXXVIII*)[104] or 4-pyrimidinyl (*XXXVIII*).

Table 6.15 lists fifty pyrimidine-containing nitrofurans of this latter type. These compounds contain a wide range of substituents at C_2 and C_5 of the pyrimidine ring. In addition, some compounds carry a substituent such as an alkyl group or a halogen atom on one of the carbon atoms of the butadienyl chain.

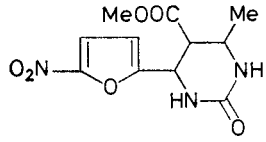
Table 6.14. 2-(5-Nitrofurylvinyl)quinoline derivatives



Compound number	R	R^1	m.p. (°C)	*References*
1	H (*N*-oxide)	H	180d. (210)	91, 95
2	4-Me	H	179	95
3	4-OMe	H	253d.	91
4	4-OMe	6-NH_2	>300	94
5	4-OMe, HCl	6-NHCOMe	>300	94
6	H	8-OMe	149	91
7	3-NH_2-4-OH	H, HCl	>300	94
8	4-OH	6-NH_2, HCl	>300	94
9	H	8-OH	187	94
10	H	5-Cl-8-OH	244–5	108
11	H	8-O·COMe	167–8	94
12	4-O:	6-NH·COMe	>300	94
13	4-O:	6-NH_2	>300	94
14	H	5-Cl-8-Cl	135–6	108
15	H	5-NO_2	267d.	91
16	H	6-NO_2	253–4	108
17	H	8-NO_2	229	91
18	4-NH_2 (lactate)	H	240d. (171–2)	91 111
19	4-NH·COMe	H	276d.	91
20	H	5-NH_2	211d.	91
21	H	5-NH·COMe	277d.	91
22	H	8-NH_2	178	91
23	H	8-NH·COMe	190	91
24	H	6-NH·COMe	280d.	91
25	4-CO·OH	H	270–1	109
26	4-CO·NH_2	6-Br	312d.	110
27	4-CO·OH	6-Br	312d.	110
28	4-CO·OH	6-NH_2, HCl	>320	94
29	4-NH_2	6-NH_2, HCl	280	111
30	H	5-NH·COMe-8-NH·COMe	279–80	94
31	3-O | 4-N=CMe	H	240	94

2. *5-Nitrofuryl heterocyclic compound*

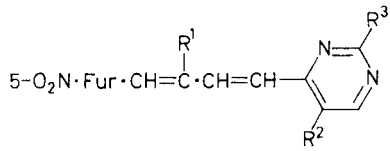
In 1958 Hull and Swain[112] synthesized 4-(5-nitrofuryl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylic acid methylester (nitractin) (*XL*) which Hurst and Hull[113] described as a compound that protects mice from *lymphogranuloma inguinale* and related infections.



(*XL*)

In this compound the nitrofuran and heterocyclic rings are joined directly.

Table 6.15. Nitrofurylbutadienylpyrimidine derivatives[96, 106, 107]



Compound number	R^1	R^2	R^3	m.p. (°C)
1	H	H	NH_2	205
2	Me	H	NH_2	215
3	Et	H	NH_2	188·5
4	Ph	H	NH_2	193·5
5	Br	H	NH_2	207
6	Cl	H	NH_2	198
7	H	Me	NH_2	232–5
8	Me	Me	NH_2	241–2
9	Ph	Me	NH_2	224–5
10	Br	Me	NH_2	210–2
11	Me	Pr	NH_2	179–80
12	H	H	Me	223
13	Me	H	Me	190–4
14	Et	H	Me	144–5
15	Cl	H	Me	242
16	H	H	Et	130·5–33
17	Me	H	Et	126–8
18	Cl	H	Et	160–2
19	Me	H	Pr	106–8
20	Me	H	Bu	109–13
21	Me	CO·OEt	Me	154–5
22	Et	CO·OEt	Me	101–5
23	Me	CO·OEt	Et	92·5–5
24	Me	H	Me	250
25	Me	Br	(Me)·CH_2·OH	239
26	Et	H	(Me)·CH_2·OH	195
27	Et	H	(Me)·CH_2·OH	83
28	Me	Cl	NH_2	232–3
29	Me	Br	NH_2	233
30	Et	Br	NH_2	221·5
31	Br	Br	NH_2	207
32	Cl	Br	NH_2	231–3
33	Me	CO·OEt	NH_2	237
34	Br	CO·OEt	NH_2	206–7
35	Cl	CO·OEt	NH_2	214–5
36	C	CO·OEt	NH_2	242–3
37	Cl	H	NH_2	161–3
38	Me	H	NHMe	205–7
39	Me	Br	NHMe	211–12·5
40	Et	Br	NHMe (α)	194–5
41	Et	Br	NHMe (β)	146–8
42	H	H	NMe_2	205–7
43	Me	H	NMe_2	178–80
44	Me	H	NHEt	155–6
45	Et	H	NHEt	152–3
46	H	H	NHBu	128–31
47	Et	H	$C_4H_8NO^a$	143–6
48	H	H	$C_5H_{10}N^b$	108–11
49	Me	H	$C_5H_{10}N^b$	127–30
50	Br	H	$C_5H_{10}N^b$	186–8

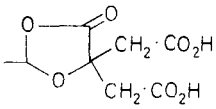
[a] morpholino [b] piperidino

In view of the biological activity of this compound, many others of a similar type were studied.

In 1959 Skagius[114] synthesized similar compounds containing thiazole (*Table 6.16*) and thiadiazole rings (*Table 6.17*). Many researchers have since focused their attention on this type of compound. The following heterocyclic rings have been joined directly at C_2 to the 5-nitrofuran nucleus:

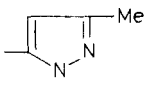
$$5\text{-}O_2N\cdot Fur\cdot R \qquad (R: \text{heterocyclic nucleus.})$$

2-(4-oxo-1,3-dioxolane-5,5-diacetic acid) (*XLI*)[115], 4-thiazole (*XLII*)[116–19] 5-(3-methylpyrazole) (*XLIII*)[120], 3-(5-methyl-1,2,4-oxadiazoline (*XLIV*)[121], 5-oxadiazolin-2-one (*XLV*)[121a], 2-(1,3,4-thiadiazole) (*XLVI*)[122], 2-imidazopyridine (*XLVII*)[123], 2-imidazopyrimidine (*XLVIII*)[123], 8-(6-chloropurine) (*XLIX*)[124], 6-(4,5-dihydropyridazine-3-one) (*L*)[125], 4-(2-oxo-1,2,3,4-tetrahydropyrimidine) (*LI*)[112], 4-uracil (*LII*)[126], 6-(3-amino-1,2,4-triazine) (*LIII*)[119], 5-(3-amino-1,2,4-triazine) (*LIV*)[119], 6-(1,2,4-triazine-3-(2H,4H)-one) (*LV*)[125], 6-(1,2,4-triazine-3,5-(2H,4H)-dione) (*LVI*)[127], 2-quinoxaline (*LVII*)[128], 2-(5-azaquinoxaline) (*LVIII*)[128], 3-(1,2,4-benzothiadiazine-(2H)-1,1-dioxide) (*LIX*)[129].



(*XLI*)

(*XLII*)



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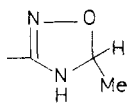
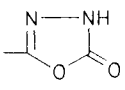
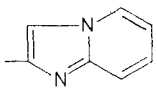


(*XLIV*)

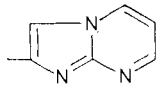


(*XLV*)

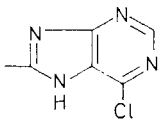
(*XLVI*)



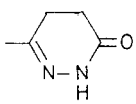
(*XLVII*)



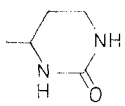
(*XLVIII*)



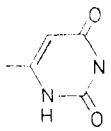
(*XLIX*)



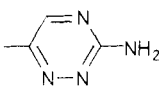
(*L*)



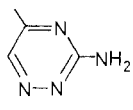
(*LI*)



(*LII*)



(*LIII*)



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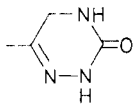
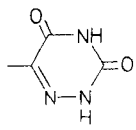


(*LV*)



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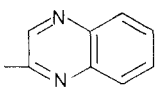
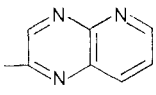
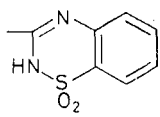


(*LVII*)

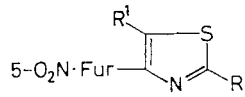


(*LVIII*)



(*LIX*)

Table 6.16. 5-Nitrofurylthiazole derivatives



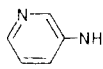
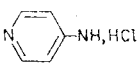
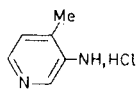
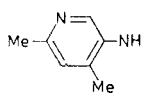
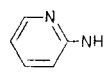
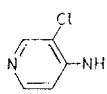
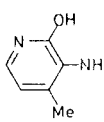
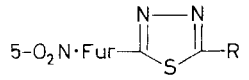
Compound number	R	R^1	m.p. (°C)	*References*
1	NH_2	H	239	117, 118
2	NH_2	Me	260	117
3	NH_2	Br	190–92	118
4	NH_2	NO_2	282–4	118
5	NHMe	H	260–1	116, 117
6	NHEt	H	158–9	116
7	NMe_3	H	168	117
8	NHPh	H	141–3	116
9	4-Me_2NPhNH	H	205	117
10	4-$Et_2N·CH_2·CH_2·NH$	H	198–200	117
11		H	174	117
12		H	250–2	117, 118
13		H	248–9	117, 118
14		H	336–7	117, 118
15		H	216–7	117, 118
16		H	212–4	117, 118
17		H	336–7	117, 118
18	Me	H	140-1, 144–5	116, 117
19	Ph	H	184–5	117, 129
20	MeCO·NH	H	295–6	118
21	EtCO·NH	H	223–5	116
22	$ClCH_2·CO·NH$	H	199–200d.	118
23	5-$O_2N·Fur·CO·NH$	H	200	118
24	$C_{15}H_{31}CONH$	H	136–7	130
25	$C_{17}H_{35}CO·NH$	H	133·5	130
26	$NH_2·C(=NH)·NH$	H	246–7	117
27	$NH_2·NH$	H	–	131
28	MeCO·NH·NH	H	228–30	131
29	NH·NH·CHO	H	214–5	131

Table 6.17. 5-Nitrofurylthiadiazole derivatives



Compound number	R	m.p. (°C)	*References*
1	NH_2	270–1d., 272	114, 132
2	NHMe	219–21d.	114, 132
3	NHEt	219–21·5d.	114, 132
4	$NHPr^n$	190–1	114, 132
5	$NHPr^i$	213–5	114, 132
6	NHBu	186–7	114
7	$NH{\cdot}CH_2Bu^t$	183–4	114
8	NHPh	253–4·5	114, 132
9	$NH{\cdot}CH_2Ph$	190·5–1·5	114, 133
10	$NH{\cdot}CO{\cdot}CH_2Cl$	300	121, 122, 133
11	C_6H_9[a]	175–8	114
12	C_5H_4N[b]	172·5–3·5	114
13	$N(COMe){\cdot}Me$	264–6	132
14	$NHCH_2{\cdot}CH{=}CH_2$	172·5–3·5d.	114, 116
15	$N(COMe)Pr^n$	139–42	132
16	$NH{\cdot}CO{\cdot}OMe$	172·5–4	114
17	$N(COMe)Pr^i$	198·5–9·5	132
18	N(COMe)Ph	244–5	122
19	SMe	174	134
20	SEt	140	134

[a] cyclohexenyl [b] 3-pyridyl

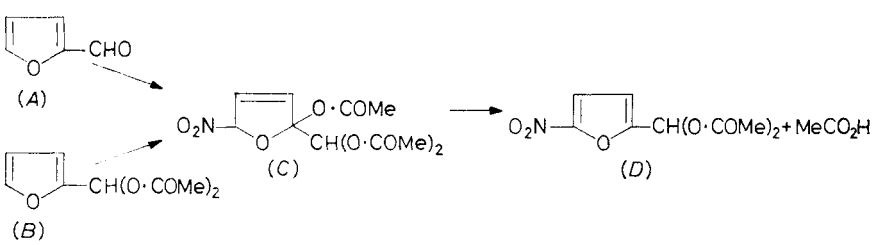
SYNTHESIS OF NITROFURANS

Synthesis of Azomethine Compounds

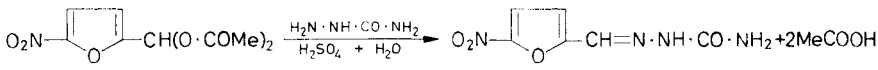
Compounds containing one nitrofuran nucleus in the molecule

Nitrofurfural and its diacetate are the commonest starting materials for the preparation of many of the therapeutically active compounds. Nitrofurfural diacetate is easily obtained by the action of acetyl nitrate on furfural(*A*) or its acetate(*B*); if acetic anhydride is in excess, a yield of 40–70 per cent can be expected. The use of strong catalysts such as sulphuric acid enhances the yield. Marquis[135] first tried to nitrate furfural and later Gilman and Wright[136] succeeded in preparing the pure 5-nitrofurfural.

The intermediate product, (*C*), when treated with a base such as trisodium phosphate or pyridine, gives the diacetate (*D*) of 5-nitrofurfural:



The azomethine derivative is generally prepared by the condensation of a substituted hydrazine with nitrofurfural in the presence of acid. For example, nitrofurazone(*E*) is formed by the interaction of 5-nitrofurfural diacetate which is hydrolysed to the active 5-nitrofurfural and semicarbazide in the presence of sulphuric acid and water. This reaction proceeds by heating at about 100°C for 5 hours.



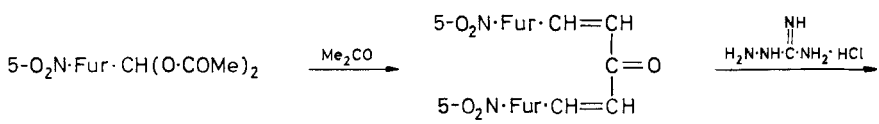
(E)

At present there are six nitrofuran azomethine compounds which are used clinically. These are synthesized by replacing semicarbazide in the preparation of nitrofurazone by hydroxylamine, aminoguanidine, 1-aminohydantoin, 3-amino-2-oxazolidinone and 3-amino-5-morpholinomethyl-2-oxazolidinone to give respectively nifuroxime[137], guanofuracin[138], nitrofurantoin[139], furazolidone[43] and furaltadone[81].

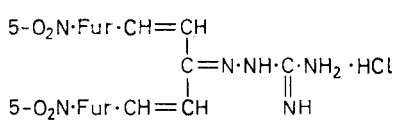
The early synthetic work on some of these azomethine nitrofuran compounds has been reviewed[140]. The nitrofurans have been discussed in a monograph[140a] on furan compounds in general.

Compounds containing two nitrofuran groups in the molecule

1,5-Bis-(5-nitro-2-furyl)-3-pentadienone amidinohydrazone hydrochloride (panazone) was prepared in 1951[3,141], and is marketed as a chemotherapeutic agent in Japan. It is necessary for the preparation of panazone to start with 1,5-bis-(5-nitro-2-furyl)pentadien-3-one. Sulphuric acid and catalyst (zinc amalgam) are added to the solution of 5-nitrofurfural diacetate in glacial acetic acid. Acetone is added dropwise to this mixture at room temperature until yellow brownish crystals of the crude bis-nitrofurylpentadienone (*F*) begin to appear. The purified ketone (*F*) in hot dioxan is added to a solution of aminoguanidine carbonate and hydrochloric acid. The mixture is heated until a precipitate of crude panazone (*G*) appears; further purification of the compound involves great difficulties.



(F)



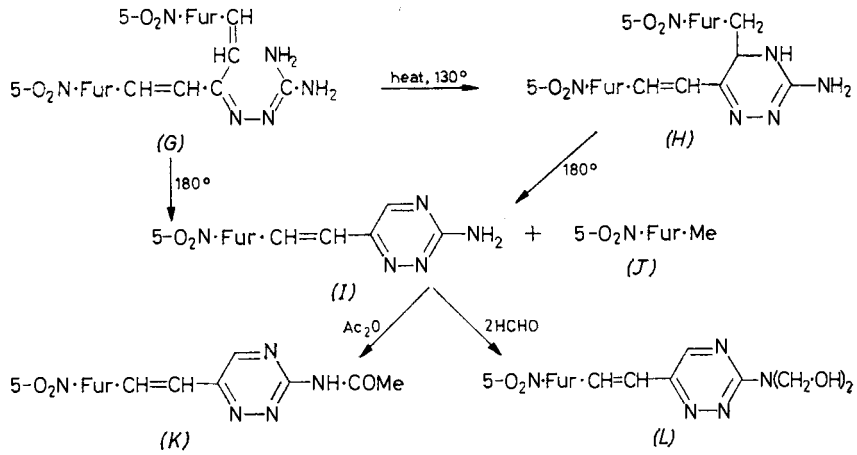
(G)

Nitrofuran Vinylogues—the Formation of Panfuran

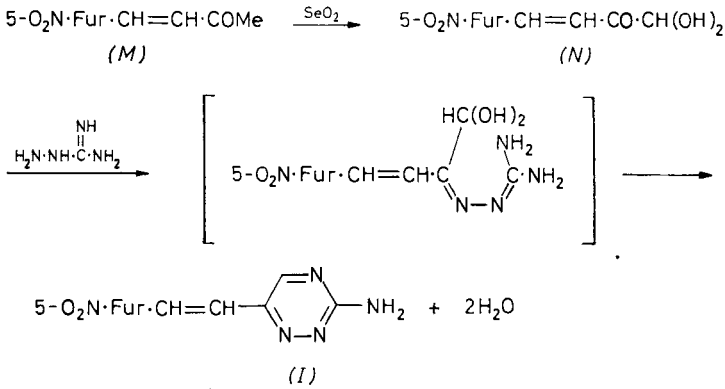
In 1961 Miura, Ikeda and Oohashi observed that when panazone is heated it is converted into a new compound which has a marked antibacterial

effect against gram positive and negative bacteria[4]. This new product is 3-amino-6-(5-nitro-2-furylvinyl)-1,2,4-triazine (panfuran)[99,100].

Panfuran is prepared by heating panazone (*G*) in an organic solvent, for example ethyleneglycol monomethylether, in the presence of sodium bicarbonate at 130–135° for 30 minutes. During this process the solution changes colour from red to yellow. After cooling, the yellow crystalline mass obtained from the solution is separated, and then dissolved again in hydrochloric acid solution. To this acidic solution an excess of ammonia is added. Panfuran base (*I*) melts at 270°. Its hydrochloride is a yellow crystalline solid melting at 234–40°; its monoacetyl derivative (*K*), which is useful as a chemotherapeutic agent, melts at 275° with decomposition. In 1964 Takai and Saikawa[100] found that the di-(hydroxymethyl) derivative (*L*) of panfuran is formed by the addition of formaldehyde. The product obtained is a yellow crystalline powder which melts at 158–61°.

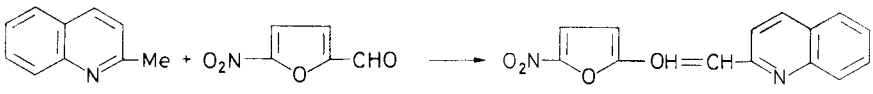


Recently Haber[142] reported that the synthesis of panfuran can be accomplished by a different process; 5-nitrofurfurylideneacetone(*M*) is converted to 5-nitrofurfurylidenepyruvaldehyde hydrate by oxidation with selenium dioxide. A suspension of the pyruvaldehyde hydrate(*N*) and aminoguanidine bicarbonate on heating in methanol gives panfuran base (*I*):



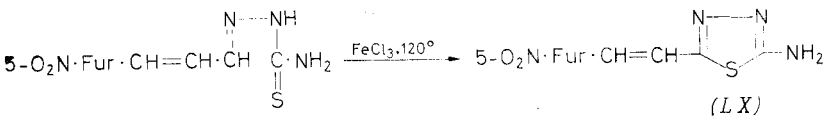
Other syntheses of the nitrofuran vinylogues involve the following two procedures[91,98]:

(1) condensation of 5-nitrofurfural and a heterocyclic compound containing an active methyl group, for example, the synthesis of 2-[(5-nitro-2-furyl)-vinyl] quinoline (*LIX*) from 5-nitrofurfural and 2-quinaldine in glacial acetic acid or in a mixture of acetic acid and acetic anhydride;



(LIX)

(2) oxidation of 3-(5-nitrofuryl) acrolein thiosemicarbazone; for example, the synthesis of 2-amino-5-[2-(5-nitro-2-furyl)vinyl]-1,3,4-thiadiazole (*LX*) from 3-(5-nitrofuryl)acrolein thiosemicarbazone.

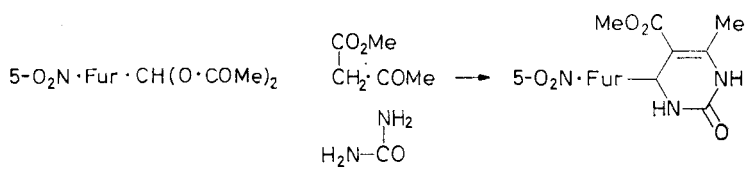


(LX)

Nitrofuryl Heterocyclic Compounds

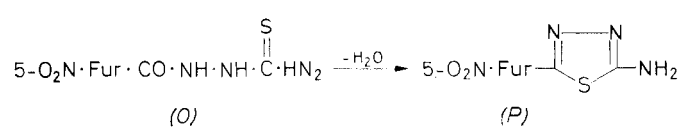
Many workers have studied the preparation of nitrofuran derivatives in which the nitrofuryl group is joined directly to the heterocyclic ring. The following examples illustrate the various procedures for synthesizing these molecules:

(1) 5-nitro-2-furfuraldehyde diacetate, urea, methyl acetoacetate, and 9 per cent methanolic hydrochloric acid solution when heated for 6 hours, and allowed to stand for one day, gave 4-(5-nitrofuryl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylic acid methyl ester (nitractin, *XL*);



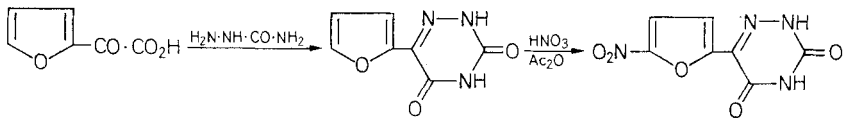
(XL)

(2) 2-amino-5-(5-nitro-2-furyl)-1,3,4-thiadiazole (*P*) was prepared by eliminating water from and simultaneous cyclization of *N*-5-nitrofuroylthiosemicarbazide (*O*)[113];



(O) *(P)*

(3) Recently Hayes[127] reported that 2-furoylformic acid (*Q*), and semicarbazide react with sodium alkoxide in propylene glycol to yield 6-(2-furyl)-1,2,4-triazine-3,5-(2,*H*4*H*)-dione (*R*). This was nitrated in acetic anhydride to give its nitrofuran derivative (*S*):



TOXICOLOGY, PHARMACOLOGY AND TOLERANCE

Toxicology

Most workers agree that the toxicity of nitrofurans, provided average dosages are given, will remain low. In therapeutic doses these compounds do not exhibit acute effects on blood pressure, respiration or heart action in man. The toxicity of nitrofurans is shown in *Table 6.18* (the LD_{50} in mice is included).

Table 6.18. Acute toxicity of nitrofurans in mice[43, 134]

Compound	*Administration*	LD_{50} (mg/kg)
Nitrofurazone	per os	745
Guanofuracin hydrochloride	per os	400
	sub.	100
Nitrofurantoin	per os	895
Panazone	per os	5,330
	sub.	3,750
	i.p.	300
Furazolidone	per os	4,543
	i.p.	300
Panfuran hydrochloride	per os	400
	sub.	300
	i.p.	50–100
Di-(hydroxymethyl) panfuran	per os	2,690
	sub.	1,602
	i.p.	1,296

The toxicity of nitrofurans varies with their solubility in water; for example, an LD_{50} of furazolidone which is slightly soluble in water changes according to the size of the crystals, and it is presumably affected by the rate of intestinal absorption.

The LD_{50} of nitrofurazone in mice ranges from 374 to 1,400 mg/kg, when the crystals range in size 375 mesh and 60 mesh. Panazone, furazolidone, and di-(hydroxymethyl)panfuran are less toxic when compared with nitrofurazone and nitrofurantoin.

Guanofuracin hydrochloride and panfuran hydrochloride are more toxic than other nitrofurans. One gram of each of the compounds will dissolve in about 100 ml. of water. Hence higher toxicity can be ascribed to greater absorption from the intestines.

Pharmacology

The pharmacological properties of nitrofurazone and nitrofurantoin were investigated by several workers[144–147]. The experimental studies[147] with various kinds of animals showed some pharmacological properties similar to those of guanofuracin hydrochloride and panfuran hydrochloride. As test devices isolated hearts of frogs, blood vessels of toads, respiration and blood pressure rates of rabbits, the central nervous system of frogs and mice, and red cell counts of rabbits were used. The results are summarized in *Table 6.19.*

Table 6.19. Pharmacological properties of nitrofurans[144, 148]

Test system	*Pharmacological effect*	
	Guanofuracin hydrochloride	*Panfuran hydrochloride*
Erythrocyte (*in vitro*)	no effect	haemolytic
Isolated heart (frog)	depress (1:500)*	depress after stimulate (1:2,000)*
Blood vessels (toad)	constriction	no effect
Blood pressure and respiration (rabbit)	rise	depress
Isolated intestine (guinea-pig)	depress the movement (1:5,000)*	depress the movement (1:25,000)
Central nervous system (frog and mouse)	stimulate (20 mg/40 g)†	stimulate (40 mg/40 g)†
Ehrlichs cancer cells (*in vitro*)	—	inhibit growth (1:800 completely)*

* Effective concentration
† Body weight of frog

Panfuran hydrochloride stimulates the central nervous system without causing paralysis. If excessive doses are administered, it causes anxiety, convulsions and stiffness shortly before death in frogs. Small dosages increase the blood pressure of rabbits, finally depression of blood pressure occurs; however, panfuran haemolyses the red cells of rabbits *in vitro* and completely inhibits the growth of Ehrlich ascites carcinoma cells *in vitro*. Guanofuracin hydrochloride contracts blood vessels, and therefore temporarily raises the blood pressure.

Blood Levels and Urinary Excretion

The achievement of adequate serum concentrations and high urinary excretion is an important factor in evaluating the merits of antibacterial compounds. This is particularly true for nitrofurantoin since it is administered most frequently by the oral route. Results of studies in this connection proved to be very favourable: 30 to 40 per cent of the administered dosages of nitrofurantoin were excreted in the urine of rats and men (*Table 6.20*).

Furazolidone, on the other hand, is excreted in the urine at a very low rate. The amount of nitrofurans found in the bloodstream or in the urine and faeces varies in accordance with the properties of each compound. The administered dose of nitrofurans cannot be totally accounted for by recovery studies. It must be assumed, therefore, that certain body tissues are capable

Table 6.20. Plasma concentration and urinary excretion of nitrofurans[149]

Compound	*Dose (oral)* mg/kg	*Plasma concentration (rat)* (%)				*Dose (oral)* mg/kg	*Urinary excretion* (%)	
		Time					*Recovered in urine*	
		0·5 h	1 h	2 h	4 h		*rat*	*man*
I Nitrofurazone	100	1·3	1·8	1·7	0·9	200	4·6	–
II Nitrofurantoin	100	4·7	3·5	3·8	4·1	200	36	32–46
III Furazolidone	100	6·5	6·2	8·7	4·2	100	1·0	trace
IV Furaltadone	100	8·5	7·6	12·5	10·5	138	3·4	1·5–2·5

of destroying them. Bender and Paul[150] reported that the concentration of nitrofurazone decreased when the compound was incubated with various mammalian tissues (intestines, liver, kidney, lung, spleen, brain and heart).

Recently, Karasaki[151] studied the blood concentration and urinary excretion of five new nitrofurans containing an oxadiazole ring and five nitrofurans which are commercially available (*Table 6.21*). These compounds were administered by stomach tube (100 mg/kg in rabbits).

Table 6.21. Concentration of nitrofurans in blood and urine of rabbit[151]

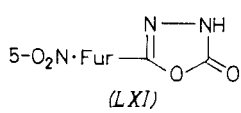
Compound tested	*Concentration in blood* (μg/mg)		*Concentration in urine* (μg/mg)		% *in urine* 6 h
	3 h	6 h	3 h	6 h	
NF-76[a]	21	10	71	48	0·38
NF-89[b]	0·6	0·9	0	0	0
NF-95[c]	0·3	0·2	19	10	0·12
NF-44[d]	3·4	5·7	10	6·3	0·15
NF-47[e]	51	34	67	27	0·8
Z-furan[f]	0	0	00	0	0
Panfuran hydrochloride	0·02	0·06	1·0	1·5	0·008
Furazolidone	0·5	0·6	0	0	0
Furaltadone	3·5	1·8	120	27	2·0
Nitrofurantoin	6·5	9·2	600	600	7·2

Dose: 100 mg/kg in stomachical application

Chemical name of compounds tested:

[a] 5-(5-O_2N·Fur)-2-methyl-1,3,4-oxadiazole.
[b] 5-[2-(5-O_2N·Fur)vinyl]-2-methyl-1,3,4-oxadiazole.
[c] 5-[2-(5-O_2N·Fur)vinyl]-1,3,4-oxadiazole.
[d] 5-[2-(5-O_2N·Fur)vinyl]-1,3,5-oxadiazolin-2-one.
[e] 5-(5-O_2N·Fur)-1,3,4-oxadiazolin-2-one.
[f] 5-Nitrofurylacrylic acid amide.

One of them, 5-(5-nitro-2-furyl)-1,3,4-oxadiazolin-2-one (*LXI*) which was synthesized by Sherman[152], shows the highest serum concentration of all tested compounds. The serum levels of nitrofurantoin are slightly less than that of the compound (*LXI*), whereas the urinary excretion of nitrofurantoin is higher in comparison to all others.

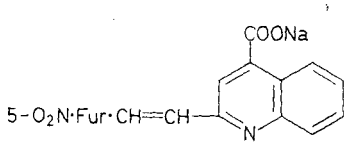


(LXI)

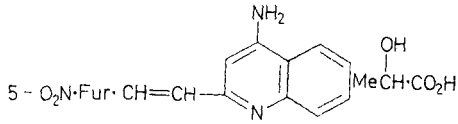
Differences in the chemical constitution of nitrofurans apparently exert an influence upon serum as well as urinary concentration.

Antitumour Activity of Nitrofurans

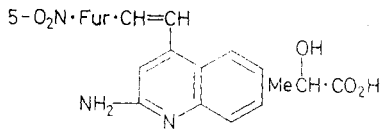
The effect of 2-[2-(5-nitro-2-furyl)vinyl]quinoline (*LIX*) and 20 other compounds on Ehrlich ascites carcinoma EY 33 in mice was tested against Miomycin C in 1964[109,111]. Pure strain healthy mice were intraperitoneally inoculated with this tumour. In general the animals will die of the accumulation of ascites after 10–19 days. Compounds are considered effective when treated mice survive 50 days after the inoculation of the tumour. The compounds found to be effective in this test were the compound (*LIX*), the sodium salt of 2-(5-nitro-2-furyl)vinylquinoline-4-carboxylic acid (*LXII*), 4-amino-2-[(5-nitro-2-furyl)vinyl]quinoline lactate (*LXIII*) and 2-amino-4-[(5-nitro-2-furyl)vinyl]quinoline lactate (*LXIV*).



(*LXII*)



(*LXIII*)



(*LXIV*)

In order to clarify the mechanism of action of these compounds, their action suppressing the dehydrogenase of *Ehrlich ascites* carcinoma cells, and the syntheses of nucleic acids and protein by *coli bacilli* was examined. It was presumed from the results that the antitumour action of the compound (*LXII*) is mainly due to the suppression of dehydrogenase action and that of compound (*LXIII*) and compound (*LXIV*) is due to the suppression of dehydrogenase action and syntheses of deoxyribonucleic acid, ribonucleic acid, and protein in tumour cells.

Development of Bacterial Resistance

It has been discovered by a number of researchers[153–158], that micro-organisms generally develop a limited degree of resistance to the nitrofurans. Micro-organisms that are transferred repeatedly into culture media containing nitrofurazone require higher minimal inhibitory concentrations later on. The increased concentration is usually 10 times, rarely 50 times higher. There are, however, exceptions; resistance of *Mycobacterium tuberculosis* to nitrofurazone and *Staphylococcus aureus* to nitrofurantoin were observed *in vitro*. Repeated transfers of *Escherichia coli* will increase the minimal inhibitory concentration of nitrofurantoin by about 35 times. Much work

has been done to investigate cross resistance to nitrofurans. The results indicated that cross resistance does not occur with nitrofurans either to antibiotics or to sulphonamides.

Kimura[159] studied the sensitivity of micro-organisms to antibiotics, panfuran hydrochloride and acetyl panfuran. He used three strains of *Staph. aureus*, five strains of *Shigella flexneri*, and one strain of *E. coli* which were isolated from various clinical sources. All strains tested had remained highly sensitive to panfuran hydrochloride and acetylpanfuran but the sensitivity to antibiotics was markedly decreased.

Development of resistance of panfuran hydrochloride to some micro-organisms by a serial transfer study *in vitro* is shown in *Figure 6.1.*

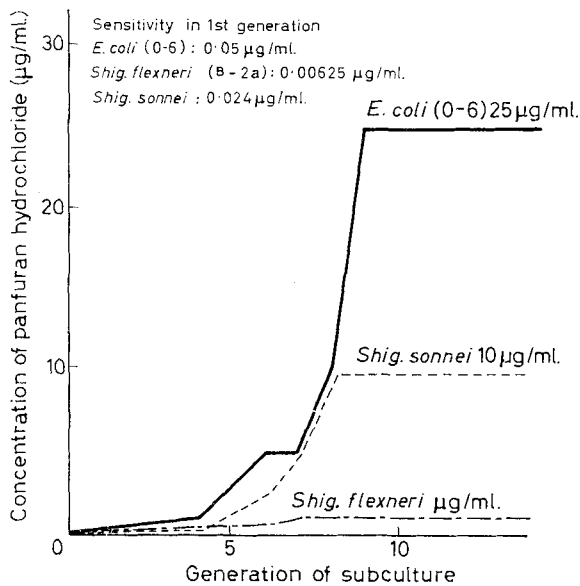


Figure 6.1. Bacterial resistance to panfuran hydrochloride[159]

After test bacteria had been transferred four times, their sensitivity to panfuran hydrochloride remained virtually unchanged. No resistance had developed *in vitro*. After fourteen transfers the sensitivity of the test bacteria *E. coli*, *Shig. sonnei*, and *Shig. flexneri* to panfuran hydrochloride decreased by 500, 400 and 15 times respectively. The results of numerous studies demonstrate that the development of nitrofuran resistance in bacteria represents the survival and multiplication of a few inherently resistant organisms rather than an induced change in all individuals. The evidence indicates also that cell permeability might be one of the reasons but not the only factor in nitrofuran resistance.

ASSAY OF NITROFURANS

In order to determine nitrofuran activity *in vitro*, the minimum inhibitory concentration (MIC) in bacterial cultures must be found by using the serial two fold dilution technique with broth as a medium. Incubation time is 24 hours at 37°C. The cup method, which is usually employed for the assay

of penicillin, is also used for the determination of nitrofuran activity. With this method the potency of nitrofurans can be measured by determining the size of the inhibition zone around the cup on the agar plate which contains the bacteria. Occasionally, the paper modification of the cup method is used. When the agar plate method with a paper disc is employed, the paper is moistened with a drop of solution of compound. The excess of solution is shaken from the paper and put on the plate. The test compound which is not soluble in water is mixed with a base of ointment; a specified quantity of this mixture is then applied to the paper disc. If the activity of the compound in urine and faeces is to be determined, it is desirable to add a 50 per cent alcohol solution which will inhibit the decomposition of nitrofurans.

A measured quantity of the test compound is administered by various routes. From the activity *in vivo* the value of ED_{50} is calculated. The chemotherapeutic index is determined from the ED_{50} and LD_{50}, which assesses the final therapeutic value of nitrofurans.

Analytical Determination of Nitrofurans

There are important indications for the use of certain nitrofurans for the prevention and cure of diseases in man and domestic animals. Recently the use of nitrofurans as an antiseptic feed additive has increased rapidly.

Since chemical analysis of nitrofurans is indispensable their characteristic ultraviolet absorption has been recommended for assay[160,161].

Reckendorf, Castringius and Spingler[162] have utilized the colorimetric and spectrophotometric determination of nitrofurantoin in the urine and serum. Stone[163], and Puglisi[164] have determined a trace of nitrofurantoin in milk by the colorimetric and spectrophotometric methods. Both procedures are based on the conversion of nitrofurantoin to 5-nitrofurfuraldehyde phenylhydrazone and are followed by the extraction and concentration on a chromatographic column. Final estimation depends upon development of a blue colour by the addition of hydramine base. Breinlich[165] studied the titrimetric, spectrophotometric and chromatographic determinations of various nitrofurans and reported satisfactory results.

Several workers[166–169] studied the polarographic behaviour of nitrofurans. Nitrofurazone in animal feed has been quantitatively assayed by polarography. Polarographic assay of nitrofurans will require more basic research before it can be used on a large scale. However, the spectrophotometric assay of nitrofurantoin has been replaced by a polarographic assay in the first U.S. P. XVI supplement (U.S. Pharmacopoeial Convention, 1962).

METABOLISM OF NITROFURANS

The nitrofurans generally prove to be stable. If the nitro group in the furan ring is reduced, biological activity will be lost. Nitrofurans which have azomethine groups (—CH=N—) or vinyl groups (—CH=CH—) lose their antimicrobial effectiveness if the —CH=N— linkage undergoes acid hydrolytic decomposition or if the —CH=CH— linkage is oxidized. It seems that the nitrofurans which are commercially available today are more or less resistant to such decomposition.

Taylor, Paul and Paul[170] recognized that the nitrofurans act as electron acceptors in the presence of xanthine oxidase in all animal tissues. Other

workers have shown that degradation also occurs in brain and muscle tissue which are known to lack xanthine oxidase[171]. Nitrofurans decompose in all animal tissues but not in the blood. Cramer[172] observed the inactivation of nitrofurazone in bacterial cultures and postulated furacinreductase as the responsible factor.

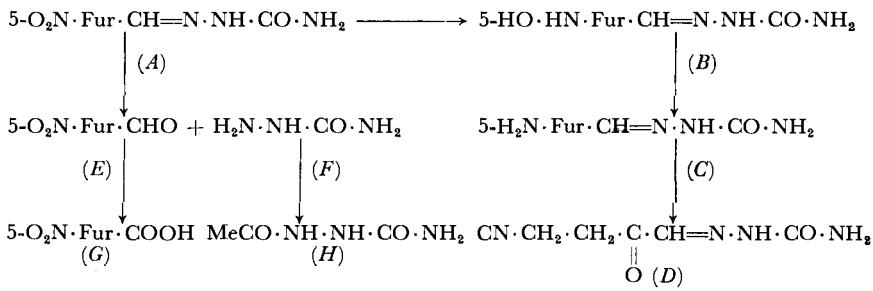
Nitro groups in the furan ring are reduced easily by various biochemical processes[173]. Nitrofurazone can function as an electron acceptor in a number of bacterial dehydrogenase systems involved in carbohydrate metabolism.

Beckett and Robinson[174–177] have conducted studies with whole cells, protoplasts, and lysed protoplasts of *Bacillus megaterium* and shown that the site of reduction of nitrofurazone is the cytoplasmic membrane rather than the cell wall or the cytoplasma.

The urine of nitrofurazone-fed animals was examined chromatographically[178,179] and was found to contain the following compounds:

(1) unchanged nitrofurazone
(2) hydroxylaminofurfuraldehyde and aminofurfuraldehyde semicarbazone
(3) 4-cyano-2-oxobutyraldehyde semicarbazone
(4) a brilliant yellow compound with an absorption peak at 412·5 mμ
(5) a brown-coloured resinous substance.

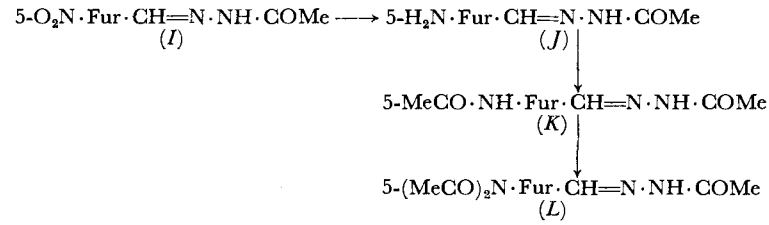
The two compounds listed under (2) could not be analysed completely. The reduction of the nitro group in nitrofurazone which was administered to animals occurs in the following manner:



Nitrofurazone (*A*) is first reduced to the hydroxylamine (*B*) which is further reduced to the amine (*C*). These chemical structures are verified by elemental and infra-red analysis. Austin[182] described the chemical properties of 4-cyano-2-oxobutyraldehyde semicarbazone (*D*) which is produced by the opening of the furan ring. On the other hand nitrofurazone yields 5-nitrofurfuraldehyde (*E*) and semicarbazide (*F*) by the acidic hydrolysis of the —CH=N— linkage. This aldehyde gives 5-nitrofuroic acid (*G*) by oxidation in animal tissues.

From the urine of animals fed with 5-nitro-2-furfuraldehyde acetylhydrazone(nihydrazone) (*I*) two end products were isolated and characterized[181] as 5-acetamido-2-furfuraldehyde acetylhydrazone (*K*) and 1,2-diacetylhydrazine. The presence of 5-amino-2-furfuraldehyde acetylhydrazone (*J*), 5-diacetylamino-2-furfuraldehyde acetylhydrazone (*L*), and 5-nitrofuroic acid (*G*) was demonstrated in the urine of the animals.

An unidentified labile, yellow metabolite with maximum absorption at 415 mμ which had been described by other workers[180] was also isolated.



It therefore appears that the decomposition of nitrofurans in animal tissue can proceed through several metabolic pathways. Recently Buzard[183] suggested that aminofurans may be converted to β-ketoglutaramic and ketoglutaric acids and the furan ring might be utilized for the biosynthesis of various normal constituents. Using chickens fed with ^{14}C labelled nihydrazone, the following labelled compounds were isolated: glucose, glutathione, glutamic acid, cysteine, glycine, aspartic acid, serine and tyrosine[184]. By the use of anion-exchange columns and paper chromatography, Tennet and Ray[185] showed the presence of more than thirty labelled compounds in the urine of swine fed with furazolidone containing ^{14}C.

In addition, nitrofurans may be metabolized to innocuous products which are reincorporated into normal body constituents.

ANTIBACTERIAL PROPERTIES *in vitro*

Bacteriostatic and Bactericidal Properties

Nitrofurazone was one of the first furan derivatives to be useful as a chemotherapeutic agent. The semicarbazones of 4-nitrobenzaldehyde, 5-nitrothiophenaldehyde, and of 5-nitropyrrolaldehyde were less effective and not suitable for clinical use[186]. Antibacterial activity is conferred on non-nitrated furans if a nitro group is introduced in the 5-position of the furan ring. Later this was recognized to be an indispensable prerequisite. Microbiologists of the Norwich Pharmacal Co. have summarized the activity against representative species of bacteria (*Table 6.22*). The six tested nitrofurans, however,

Table 6.22. Antibacterial activity *in vitro* of nitrofurans[187]: minimal inhibiting concentrations (μg/ml.), 24 hours

Compound	*E. coli*	*Staph. aureus*	*Sal. typosa*	*Pr. vulgaris*	*Ps. aeruginosa*	*Strept. pyogenes*	*Aero. aerogenes*
Nitrofurazone	6	5	10	40	200	10	13
Nitrofurantoin	3	12	12	100	>700	6	100
Furazolidone	1·5	5	0·7	55	> 99	>55	5
Furaltadone	2	2	22	390	>390	45	172
Nifuroxime	10	37	1	194	194	23	No data
Nitrofuryl methylether	14	57	17	480	480	22	No data

are consistently effective in low concentration against five of the seven tested species of micro-organisms. It is noticeable that one of them, i.e. furazolidone,

demonstrates strong antimicrobial activity against *Salmonella typhosa* and *E. coli* in a concentration of 1:1,000,000 μg/ml.

Moreover, furazolidone[188] demonstrates strong bacteriostatic action against a wide variety of micro-organisms (except against *Proteus vulgaris*, *Pseudomonas aeruginosa* and *Streptococcus pyogenes*). Nifuroxime is primarily fungicidal and particularly effective against *Candida* (*Monilia*) *albicans*. Nitrofurfuryl methyl ether is effective against a variety of pathogenic fungi[189].

It is interesting that many gram negative and gram positive bacteria, numerous protozoa, and even some large viruses are susceptible to various nitrofurans *in vitro*. In 1947 Stillman and Scott[190] synthesized 5-nitrofurfurylidene-aminoguanidine sulphate by the condensation of nitrofurfural and aminoguanidine sulphate. In 1949 the hydrochloride salt was synthesized and called guanofuracin hydrochloride. It is soluble in water in a concentration of 1:100, and its antibacterial activity is similar to that of nitrofurazone[138,191].

Table 6.23 shows the bacteriostatic and bactericidal activity of three new nitrofurans in comparison with furazolidone and three antibiotics.

Table 6.23. Comparison of antibacterial activity of nitrofuran derivatives[144] and antibiotics *in vitro*

	1. *Bacteriostatic activity* 2. *Bactericidal activity*	*Maximum dilution* (μg/ml.) *at which compounds exhibit activity*				
		Shig. dysenteriae (Komagome BIII strain)	*Sal. typhi.* (O)	*E. coli* (Gakusei strain)	*Staph. aureus* (Terashima)	*Strep. haemolyticus* (S-type)
Panfuran hydrochloride	1	0·10	0·05	0·03	0·19	0·39
	2	0·10	0·05	0·05	0·39	0·78
Guanofuracin hydrochloride	1	7·82	7·82	7·82	7·82	7·82
	2	7·82	7·82	7·82	7·82	7·82
Panazone	1	62·50	125·0	62·50	0·12	0·98
	2	125·0	125·0	125·0	0·49	0·98
Furazolidone	1	0·98	0·98	0·98	1·95	62·50
	2	1·95	0·98	1·95	1·95	62·50
Chloramphenicol	1	0·98	1·95	1·95	3·91	0·38
	2	15·62	31·25	62·50	31·25	1·95
Tetracycline	1	1·95	1·56	3·91	0·98	0·49
	2	15·62	2·50	62·50	7·82	1·95
Kanamycin	1	3·91	1·56	15·62	0·49	31·25
	2	15·62	1·56	15·62	0·98	31·25

Panfuran hydrochloride exhibits excellent antimicrobial activity against all test micro-organisms, especially against *E. coli* and *Shig. dysenteriae*. Nitrofurans not only possess bacteriostatic but also bactericidal effects against many micro-organisms. The concentration of chloramphenicol and tetracycline required for bactericidal action is much higher than that necessary for a bacteriostatic effect[86,101,192]. The activity of acyl derivatives and alkyl derivatives of panfuran compare with that of furazolidone as follows: the three compounds tested, namely monoacetyl-, monochloroactyl- and diacetyl-derivatives of panfuran, are more active than furazolidone, while they are less active than panfuran base. Decanoylpanfuran which is soluble

in fat, has demonstrated good effectiveness *in vitro*. Monomethylpanfuran as well as di-(hydroxymethyl)panfuran inhibits equally well the growth of bacteria. But the antimicrobial activity of both is less than that of panfuran hydrochloride. Other workers[193] reported that di-(hydroxymethyl)panfuran was more effective than panfuran base against various micro-organisms *in vitro*. Nitrofuran vinylogues containing a heterocyclic ring in the molecule, demonstrate strong activity against gram negative and gram positive bacteria *in vitro*[194].

Factors Affecting Activity

The inhibitory activity of nitrofurans on the growth of bacteria in broth or synthetic media is not significantly reduced by adding blood or serum. The antibacterial activity of certain nonionized nitrofurans (nitrofurazone, furazolidone) is not affected by the pH of the medium. Ionized nitrofurans (nitrofurantoin, nifuroxime, and furaltadone) are affected by the pH of the medium to some degree. Panfuran hydrochloride becomes less effective by adding blood or deoxycholic acid; the reduction ranged from one-half to one-fifth. However, the activity of panfuran decreases considerably by adding compounds which contain SH-groups in the molecule, namely glutathione, cysteine, sodium hyposulphite and sodium thioglycolate[195].

Panfuran readily forms an adduct with cysteine nydrochloride in water. This adduct has no antibacterial activity[105].

A study of the binding of nitrofurans to plasma proteins showed that[119] nonionized nitrofurans were bound to the plasma proteins to some extent (about 30 per cent), the anionic nitrofurans to a greater extent (nitrofurantoin about 50 per cent) and cationic nitrofurans to a relatively slight extent (furaltadone about 10 per cent). Panfuran was bound to plasma proteins to a slight extent, and to the erythrocytes to about 20 per cent. Also, the decrease of antibacterial activity of panfuran by animal tissues was observed[195]. These tissues are stomach, intestines, liver, kidney and testicle. The inactivation of the antibacterial activity of panfuran is counteracted by adding vitamin K_3 and anthraquinone.

Antibacterial Spectrum of Nitrofurans

The antibacterial activity of nitrofurazone and related compounds was investigated by several workers against a wide variety of micro-organisms. In this chapter the antibacterial spectrum only of panfuran will be given. Kimura[195] reported that panfuran had a high order of antibacterial activity against various micro-organisms (*Table 6.24*).

It is effective against gram positive and gram negative bacteria, and fungi belonging to the *Trychophyton* and *Cryptococcus* genera, except the strain of *Pseudomonas aeroginoses* and *candida*. The *in vitro* spectrum of panfuran closely resembles that of nitrofurazone.

ANTIBACTERIAL PROPERTIES *in vivo*

Studies in Experimental Animals

It is understood that nitrofuran derivatives exhibit an outstanding inhibition against various micro-organisms *in vitro*, although the cure rate of infected animals remains rather low.

Table 6.24. Antimicrobial spectrum of panfuran hydrochloride[195]

Micro-organism	*Minimum inhibitory concentration* (μg/ml.) (24 h)
Staphylococcus aureus (209p)	0·25
Staphylococcus aureus (Terashime)	0·25
Diplococcus pneumoniae	0·5
Streptococcus pyogenes (S-43-M)	0·25
Streptococcus pyogenes (variant-7)	0·5
Bacillus subtillis (PCL)	0·5
Corynebacterium diphtheriae	0·25
Escherichia coli (0–6)	0·3
Escherichia coli (0–9)	0·5
Escherichia coli (0–18)	0·05
Escherichia coli (0–55)	0·05
Escherichia freundii	0·25
Aerobacter aerogenes	0·5
Arizona	0·05
Alkaligenes dispar	0·1
Klebsiella rhinoseromatis	1·0
Proteus vulgaris	1·0
Shigella dysenteriae (A-2)	0·1
Shigella dysenteriae (A-5)	0·1
Shigella dysenteriae (A-7)	0·25
Shigella flexneri (B-2a)	0·1
Shigella flexneri (B-2h)	0·1
Shigella sonnei (D)	0·05
Salmonella typhi (T-63)	0·1
Salmonella paratyphi A	0·1
Salmonella paratyphi B	0·1
Salmonella entritidis	0·01
Pseudomonas aeruginosa	20·0
Pseudomonas enteritis (Kanagawa 2-XII)	0·1
Pseudomonas enteritis (II)	0·01
Pseudomonas enteritis (III)	0·025
Pseudomonas enteritis (IV)	0·025
Pseudomonas enteritis (VI)	0·005
Pseudomonas enteritis (VII)	0·01
Pseudomonas enteritis (VIII)	0·025
Pseudomonas enteritis (IX)	0·025
Pseudomonas enteritis (Kanagawa 1-II)	0·005
Pseudomonas enteritis (XI)	0·025
Pseudomonas enteritis (XII)	0·025
Pseudomonas enteritis (XIII)	0·1
Pseudomonas enteritis (XIV)	0·05
Pseudomonas enteritis (Kanagawa 3-XIII)	0·005
Pseudomonas enteritis (N_4-II)	0·025
Pseudomonas enteritis (XVIII)	0·025
Candida albicans	10·0
Candida perakrusei	50·0
Candida tropicalis	25·0
Candida stelletoides	5·0
Candida guilliermendi	50·0
Candida krusei	10·0
Trichophyton mentagrophytes (Kameda)	2·5
Trichophyton interdigitales (Yoshida)	5·0
Trichophyton rubrum (Fujisawa)	0·6
Trichophyton rubrum (Iura)	1·25
Cryptococcus neoformans	5·0

The broth dilution method was used in these studies. *Pseudomonas* organisms were cultivated in 1 per cent peptone, 3 per cent NaCl medium, and *Dipl. pneumoia, Str. pyogenes* and *Coli. Diphtheriae* were cultivated on heart infusion broth containing 0·5 per cent glucose. All other bacteria were cultivated on heart infusion broth. *Fungi* were grown in Sabouraud's medium.

Dodd[196] reported that nitrofurazone was effective against infections with *Staph. aureus*, *Strep. pyogenes*, *Sal. schottmuelleri*, and *Sal. typhimurium*. It is not effective against *Diplococcus pneumoniae*. The antibacterial activity of other nitrofurans *in vivo* is shown in *Table 6.25*.

Table 6.25. Chemotherapeutic activity of nitrofurans (oral dosage)[197] against *Staph. aureus* and *Sal. typhosa* in albino mice

Compound	*Staph. aureus*		*Sal. typhosa*	
	Dose (mg/kg)	*Survival* (%)	*Dose* (mg/kg)	*Survival* (%)
Nitrofurazone	210	50[b]	93	50[b]
Nitrofurantoin	210	10	210	10
Furazolidone	22	50[b]	30	50[b]
Furaltadone	22	50[b]	75	70
Nifuroxime	no data	—	45[c]	20
Nitrofurfurylmethyl ether	no data	—	350[d]	60

[b] ED_{50}.
[c] 3 doses : 14 mg/kg at 30 minutes, 4·5 and 8 hours. (No single dose data.)
[d] 100 mg/kg at 30 minutes, followed by 50 mg/kg at 3, 6, 9, 12 and 24 hours. (No single dose data.)

All test compounds are effective against systemic infection with *Sal. typhosa*. Nitrofurazone, furazolidone, and furaltadone are also effective against infection with *Staph. aureus*. In 1962 the activity of panfuran *in vivo* was determined against infections with *Strep. pyogenes*[144]. Mice were inoculated intraperitoneally with a dose 1,000 times higher than the MLD of bacteria. Thereafter, panfuran and similar compounds were administered subcutaneously at 3, 24, 48, and 72 hours after inoculation. The results prove that panfuran is effective against *Strep. pyogenes* infections.

Its effectiveness, however, seems to be less than that of the tetracyclines. Kimura[193] proved di-(hydroxymethyl)panfuran to be effective *in vivo* against *Strep. pyogenes* infections as the following *Table 6.26* shows. The three tested compounds exhibit the same prophylactic activity.

Table 6.26. Prophylactic effects of acetylpanfuran and di-(hydroxymethyl)panfuran against *Streptococcus pyogenes* infection in mice[198]

Compounds tested	320 mg/kg	160 mg/kg	80 mg/kg	40 mg/kg	ED_{50} mg/kg
Di-(hydroxymethyl)panfuran	10/12	7/12	3/12	3/12	80–160
Acetylpanfuran	7/8	5/8	3/8	1/8	80–160
Tetracycline	4/4	4/4	0/4	0/4	80–160

At 30 minutes before the inoculation, the compounds were orally administered. Control animals died from the infection.
Virulence = 1 : 1,000 i.p.

Trypanocidal Nitrofurans

The most important trypanocidal furan is nitrofurazone which will cure *T. equiperdum* infections in laboratory animals. It is effective against *T. equiperdum* in 20 hours at a concentration of 1·2 μg/ml. and *T. rhodensiense in vitro*.

In vivo curative dose (CD_{50}) for *T. equiperdum* in mice is 248 mg/kg if the

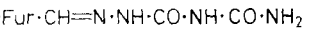
drug is administered subcutaneously (single dose[199]). A single intramuscular dose of 50–150 mg/kg cured 16 of 32 guinea-pigs infected with various strains of *T. gambiense*.

A recently isolated strain of *T. rhodensiense* in mice is not cured intraperitonally[200]. In mice infected with *T. cruzi* the infection was inhibited by nitrofurazone and the following three compounds[201,202]:

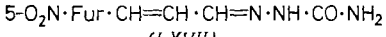
(1) 5-nitro-2-furfurylideneaminobiuret (*LXV*);

(2) 5-nitro-2-furfuraldehydetrimethylammoniumacetylhydrazone chloride (*LXVI*);

(3) 5-nitro-2-furylnacrolein semicarbazone (*LXVII*).



(LXV)



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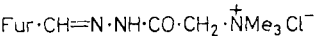
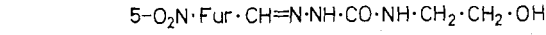
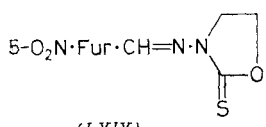


(LXVI)

Trypanosomes are not resistant to nitrofurazone. Furaltadone is ineffective against infections with *T. rhodensiense*[203]. Both the 2-hydroxyethyl semicarbazone (*LXVIII*) and the thioxazolidone (*LXIX*) derivatives of 5-nitrofuraldehyde have some activity in mice infected with trypanosoma[202,204].



(XVIII)



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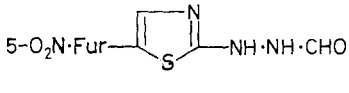


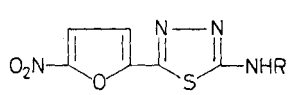
(LXX)

Some Compounds under Clinical Investigation against Bacterial Infections

Recently it was reported that the following compounds were under clinical trial:

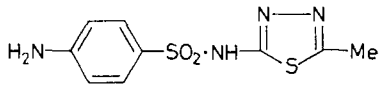
(1) Through the investigation of Holper[205] on the antibacterial effectiveness of 5-(formylhydrazino)-2-(5-nitro-2-furyl)thiazole (*LXX*) which was synthesized by Sherman and Dickson[205a] it is proved that this compound, *in vitro*, is about twice as effective against gram positive bacteria and about four times against gram negative bacteria, as is nitrofurantoin. Systemic infections in mice with *Staph. aureus*, *Strep. pyogenes*, *E. coli*, and *Sal. typhimurium*, respond well when it is administered orally or subcutaneously. It is inactive *in vivo* against *B. aeruginosa*, *Pr. vulgaris*, and *Diplo. pneumoniae*.

(2) The antibacterial action of several new nitrofurans *in vitro* against a number of organisms has been reported by Skagius[206]. These data indicate that 5-(5-nitro-2-furyl)-2-amino-1,3,4-thiadiazole (NF-475, ASA-140, Ph/778, *LXXI*) and its formyl derivative (*LXXII*) have been recommended for further study in the treatment of intestinal infections of various aetiology.

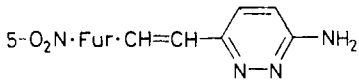


(*LXXI*, R = H)
(*LXXII*, R = CHO)

(3) The activity *in vivo* of a nitrofuran, 2-amino-5-[2-(5-nitro-2-furyl)-vinyl]-1,3,4-thiadiazole (*LX*) was tested against *Strep. pyogenes*-infected mice[194]. The *in vivo* effectiveness against streptococcal infection of this compound is superior to that of sulphamethizole (*LXXIII*) which is widely used in urinary tract infections. The same compound was synthesized independently by workers of Boehringer & Soehne C.m.b.H.[207] and its clinical effectiveness on urinary tract infections was studied.



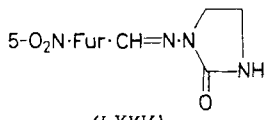
(LXXIII)



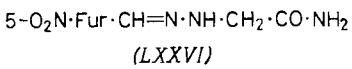
(LXXIV)

(4) It was also reported that topical use of 6-amino-1-[(5-nitro-2-furyl)-vinyl]pyridazine (NF-1002, HB-115, nifurprazine, Carofur, *LXXIV*) for soluble dressing and soluble powder gave very satisfactory results in over 400 cases. The compound was effective in the presence of pus, blood and serum and facilitated wound healing without damaging tissues.

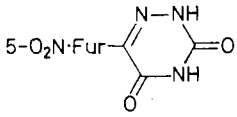
(5) 1-(5-Nitrofurfurylideneamino)-2-imidazolidone (Oxafuradene, NF-246, *LXXV*) has demonstrated a high order of antibacterial activity in the urine and blood of humans[208]. Beside this compound, the chemotherapeutic



(LXXV)



(LXXVI)



(LXXVII)

value of 5-nitrofurfurylidenehydrazinoacetamide (*LXXVI*) and its acyl derivatives were reported by the same workers.

(6) 6-(5-Nitro-2-furyl)1-, 2, 4-triazine-3, 5(2*H*, 4*H*)-dione (NF-477, *LXXVII*) was prepared and evaluated by Hayes (1964)[127]. Whereas the antibacterial spectrum of this compound is superior, urinary excretion and acute toxicity are comparable to that of nitrofurantoin. Since the incidence of vomiting in dogs was much lower than with nitrofurantoin, this compound merits further study as a urinary tract antibacterial agent.

Therapeutic Uses of Nitrofurans

Nitrofurazone

This compound is available in more than a dozen formulations. Its primary use is as a topical agent; oral application for the treatment of certain tropical diseases and seminoma plays an insignificant yet important role.

Nitrofurazone was the first product which the originators of nitrofurans, the Norwich Pharmacal Company, made available for general use through Eaton Laboratories in 1945. A year earlier the product stood its first test during the invasion of France and was beneficial in the treatment of infected battle wounds. Despite extensive use all over the world, there has been virtually no increase in bacterial resistance. Nitrofurazone exhibits bactericidal action which is not decreased by the presence of blood, pus or serum[209]. Although effective against a great variety of gram negative and gram positive micro-organisms, only those commonly found in surface infections will be mentioned here[209].

(1) Gram positive	*Staphylococcus albus*
	Staphylococcus aureus
	Streptococcus pyogenes (*haemolyticus*)
(2) Gram negative	*Proteus vulgaris*
	Pseudomonas aeruginosa
	E. coli

A recent survey by Eaton Laboratories[209] indicates that sensitization to nitrofurazone occurs at a rate of about 1·2 per cent.

The report covers more than 11,000 patients and includes those with chronic ulcers and infected ecthyma who are particularly prone to sensitization. In surgical practice the incidence is only 0·5 per cent. Whenever sensitization occurs, all symptoms and signs quickly disappear as soon as the drug has been discontinued. The risk of sensitization can be significantly decreased by applying nitrofurazone preparations only to infected areas and not to surrounding tissue. When large ulcers, varicose or decubital are treated with nitrofurazone, the sensitive skin along the lesion should be protected with zinc oxide ointment or paste. To prevent adherence of the bandage in skin grafts, it is advisable to use paper or petrolatum gauze over nitrofurazone-impregnated dressing. Nitrofurazone-soluble powder also contains 0·2 per cent of the compound, which is dissolved in polyethylene glycols. The powder is used for prophylaxis and treatment of surface infections whenever its use is more convenient than that of dressing or solution. Besides its use in the treatment of wounds and infected dermatological lesions, nitrofurazone is available for the treatment of cervicovaginal infections and for the prevention of infections in cervicovaginal surgery. Other formulations include nitrofurazone urethral inserts for the treatment of menopausal and postmenopausal urethritis, ophthalmic liquid and ophthalmic ointment for infections of the conjunctiva and cornea, ear drops which also provide local analgesia and antifungal activity, and nose drops for the relief of nasal congestion.

The application of any of its formulations for less than 24 hours is probably of little value. On the other hand, nitrofurazone should not be applied for more than 5–7 days, although most infections will have been controlled by that time. In view of the low surface tension and the product's penetration of pouches and fissures, the so-called delayed re-dressing technique, i.e. the use of a single application, can be recommended for topical nitrofurazone[209]. Although sensitization may occur occasionally, there have been few reports on clinical toxicity during recent years. Loriia[210] reported a severe allergic reaction in a woman with otitis externa. This side effect occurred after the application of nitrofurazone powder to the external auditory meatus. Shortly thereafter, pruritus and a burning sensation of the entire body with maceration and ulceration of some articular surfaces occurred. It must be noted, however, that this patient had demonstrated an allergic reaction one year prior to this incident when she used nitrofurazone solution as a throat gargle.

The most widely used formulation of nitrofurazone is the 'soluble dressing', a preparation suitable for surgical as well as dermatological infections. In burns, it is applied for treatment as well as for the prevention of infections. Pre-operative application is advised for skin grafting whereas nitrofurazone is used post-operatively on grafts as well as on donor's sites. Due to the high water-solubility there will be no interference with successful 'takes'.

Bilich[211] reported excellent results in burns in children when he added novocain and cod-liver oil to nitrofurazone. With this treatment he observed only one ineffective case in 56 patients. The more recent literature[212–216] confirms previous reports on the clinical usefulness of nitrofurazone. In comparative studies in dermatology[217] a cure rate of 65·2 per cent was reported, whereas Stauber[218] achieved satisfactory results in 90 per cent of his 95 patients with pyogenic dermatoses. In another dermatological investigation[219] a mixture of nitrofurazone, hydrocortisone and Vitamins A and D resulted in a cure rate of 92·3 per cent. For the treatment of burns, Ingham[220] used a nitrofurazone-soluble aerosol which proved to be more effective than four other aerosols with which he compared nitrofurazone. In Russia, nitrofurazone is also used as a constituent in a paste which protects the hands of workmen from penetration by industrial impurities and from irritation caused by industrial oils[221].

When a nitrofurazone solution in a concentration of 1:5,000 is used 2 hours before each meal to irrigate the tonsils of patients with tonsillitis, a significant decrease in the tonsillar bacterial count does not ensue[222]. Bladder infections, on the other hand, were noted to respond well to irrigations with a 0·4 per cent nitrofurazone solution[223], although it took 30 days until the principal infectious organisms were suppressed. Since 100 mg of nitrofurantoin were given three times daily at the same time, the effect which the bladder irrigations exercised is dubious. The beneficial use of oral nitrofurazone in malignant teratoma of the testicle has recently been reaffirmed by Rosenblatt[224]. After the surgical removal of the primary tumour, he noted complete disappearance of metastases. However, nitrofurazone should only be used as a final resource because of its neurotoxicity.

Another indication for the oral or even intravenous use of nitrofurazone is Chagas disease which occurs almost exclusively in tropical latitudes.

Four of ten patients who were treated with oral dosages of nitrofurazone ranging from 10 to 30 mg/kg daily for several weeks, received progressively increasing dosages until evidence of peripheral neuropathy appeared during the third week. Other patients on 10 mg/kg of nitrofurazone daily in conjunction with Vitamin B complex for 60 days showed no evidence of neurological side effects[225,226]. The clinical usefulness of nitrofurazone in the treatment of this tropical disease as evidenced by a negative complement fixation test for *Trypanosoma cruzi* seems to be beyond doubt, although clinical, electro-cardiographic and radiological changes due to the activity of the drug were not significant. In a subsequent study peripheral neuritis, nausea and vomiting were cited as untoward reactions[227].

Cancado, Marra and Brener[228] also used 10 mg/kg daily of nitrofurazone perorally for 10 to 34 days, but in this group of five patients, therapy had to be interrupted in all cases due to the appearance of peripheral neuritis. While topical application of nitrofurazone is usually not accompanied by any side effects, and the use of nitrofurazone inserts in patients with urethritis can also be considered a safe procedure[229], trouble may ensue as soon as the drug is given orally or parenterally. Besides the rare occurrence of haemolytic anaemia[230], the most prominent untoward effect seems to be peripheral neuritis[231].

Nitrofurantoin

This compound was the first nitrofuran which was recommended for peroral use. In 1952, eight years after the report on nitrofurazone by Dodd and Stillman[1], successful clinical trials began, documented by the first presentation in April of the following year by Norfleet, Beamer and Carpenter[232]. Nitrofurantoin was available soon thereafter for the treatment of urinary tract infections and only a selection of the hundreds of papers which have been published since can be summarized here. Looking back over the past decade and remembering that hundreds of nitrofurans have been tested for the treatment of urinary tract infections during this period, the decision to progress with nitrofurantoin more than a dozen years ago appears to have been a particularly fortunate one in that this product possesses nearly all the important properties required of a good drug in this field. The chief requirement is rapid urinary excretion of a high percentage of the ingested dose. Nitrofurantoin fulfils this prerequisite ideally, in that approximately one-third of the drug can be recovered in the urine. In order to avoid precipitation of an antibacterial agent in the urinary tract it must be stable and soluble in an acid, neutral or even an alkaline environment; moreover, if changes of pH occur, the effectiveness of the compound should not be impaired. In view of the chronic infections which have to be treated with urinary tract antiseptics, it is essential that a therapeutic agent can be administered over a longer period of time and, furthermore, that no increase of bacterial resistance occurs. Nitrofurantoin also fulfills these requirements. Its limitation in clinical use is the incidence of side effects, namely nausea and vomiting which occur in some individuals despite precautionary measures.

Of the four major causative agents of urinary tract infections, namely *E. coli*, *Enterococci*, *Staphylococci* and *Proteus*, the first three are highly sensitive

to nitrofurantoin. Legler[233] tested the sensitivity of 2,154 strains of micro-organisms which were isolated from the urine of patients with urinary tract infections and his work has been largely confirmed by other researchers. Their results are shown in *Table 6.27.*

Table 6.27. Sensitivity of bacteria to nitrofurantoin

Micro-organism	*Sensitivity* (%)			
	Legler[233]	*Grothues-mann*[234]	*Thompson and Rae*[235]	*Naumann*[236]
Escherichia coli	96	78	91	92
Aerobacter aerogenes	83			
Proteus vulgaris	27	27	72	71
Enterococci	99	81	94	99
Staphylococcus aureus	92	84	100	
Staphylococcus albus	99			

The sensitivity of the organisms to nitrofurantoin and five other antibiotics and chemotherapeutic agents was compared and, except for the greater sensitivity of *Proteus* to nalidixic acid, none of the test compounds surpassed the results achieved with nitrofurantoin. An interesting study by King[237] proved that cultures of *E. coli* and *Staph. aureus* taken at the Cleveland Clinic in the U.S. between 1956 and 1961 showed increased sensitivity to nitrofurantoin but increased resistance to dihydrostreptomycin, chloramphenicol and neomycin.

The potential value of nitrofurantoin as a urinary tract drug was definitely established in 1953, when Paul, Hayes and Bender reported their earliest results on excretion[238]. Meanwhile an abundance of literature relating to the experimental aspects has been published, and three of these, although not strictly part of a clinical description, have an indirect bearing on the therapeutic use of nitrofurantoin. The first by Voemel and Spingler[239] showed that the rate of excretion and the total amount of nitrofurantoin excreted were approximately the same for all formulations (tablets, syrup, pearls and injections) of the sodium salt. Secondly, measurement of the endogenous creatinine clearance prior to and during the administration of nitrofurantoin in a number of normal children, who had received 5 mg/kg of the drug for 7 days, showed that the plasma creatinine remained within normal limits[240]. A third study[241] indicates the need for antimicrobial assay of the renal lymph in addition to urine and blood levels when determining the effectiveness of any urinary tract therapeutic agent. Colorimetric determinations were made of sodium nitrofurantoin in the renal lymph of 17 dogs after giving 5 to 10 mg/kg of the drug by stomach tube. One and a half to two hours after the infusion, a two to three fold increase in lymph concentrations of nitrofurantoin was observed. The renal lymph was bacteriologically active against *E. coli*, indicating the effectiveness within the renal tissue.

Besides reports[242–248] demonstrating the clinical effectiveness of nitrofurantoin when given in doses ranging from 5 to 8 mg/kg daily over periods varying from 10 to 42 days, additional studies show that prophylactic

application of nitrofurantoin in pregnant women with bacteriuria can significantly reduce the incidence of pyelonephritis. Ognibene, Mullin and Schreiber[249] reported bacteriuria in 28 of 600 pregnant women; each of them received 150 mg of nitrofurantoin daily throughout the remainder of pregnancy and for 8 weeks postpartum with the result that not a single case of acute pyelonephritis occurred. LeBlanc and McGanity[250] found the incidence of significant bacteriuria to be 9·7 per cent in 1,325 pregnant women. They may have discovered a connection between bacteriuria and prematurity, namely that patients with bacteriuria who were not treated with a urinary antiseptic such as nitrofurantoin had the highest prematurity rate. The same findings in regard to pyelonephritis as well as to prematurity were confirmed by Semmens[251], LeBlanc and McGanity[252] and Schreiber[253].

The prophylactic use of nitrofurantoin has also been of great value for the prevention of infections after cystoscopy and surgical procedures. Milberg and Hermann[254] reported the occurrence of an infection in only 19 per cent compared with 70 per cent or more when no drug had been given prophylactically.

Williams[255] has also used the drug for the prevention of bladder infections in alternate patients who had undergone vaginal operations. Whereas 80 per cent of his control group developed such infections, mostly due to *E. coli*, only 10 per cent of the treated group showed evidence of this complication. Since postoperative urinary tract infection is common whenever an indwelling catheter is used, nitrofurantoin bladder instillations twice daily have been employed to reduce the incidence by almost 40 per cent, as control studies have demonstrated[256]. Although topical use of the drug is not common, a few reports[257–259] have appeared in the literature over the past few years.

The treatment of recurrent pyelonephritis, which is often due to urinary stasis in the renal tubuli, has always been a challenge to the physician. A plan to prevent infection with some sort of treatment extending beyond the acute stage has been realized by a number of researchers, most notably by Haschek in Vienna[260–263]. He has used 100–200 mg of nitrofurantoin daily for as long as five years, emphasizing that this dosage was found to be effective in reducing the frequency of relapses. Recently, he published results on the treatment of 50 patients with chronic pyelonephritis. A number of other clinicians have meanwhile confirmed the results of longterm treatment. Orsten[264] used 50–150 mg/day up to two years and successfully suppressed clinical manifestations of this chronic disease. Kasanen[265] recorded treatment up to one year and reported a low incidence of relapses in comparison with patients treated for a period of three months. Other papers[266–268] confirmed the positive results of longterm treatment although their courses were somewhat shorter. Szendei[269] treated a physician with pyelonephritis for more than three years with 50 to 100 mg of nitrofurantoin which suppressed the infection despite multiple daily catheterizations.

Whenever oral administration of nitrofurantoin does not seem feasible, the sodium salt can be administered intravenously (180 mg twice a day for adults) for the suppression of urinary tract infections[270–273]. A number of clinicians have used nitrofurantoin sodium in urology as well as for the treatment of systemic and pulmonary infections[274–281].

Intramuscular administration of nitrofurantoin sodium may also be successful[282]; in metastatic seminoma, the drug apparently decreases the size of lung metastases thus improving the patient's condition[283]. Owing to the low blood levels which are achieved with nitrofurantoin, all indications for its use aside from urinary tract infections have meanwhile been abandoned; hence nitrofurantoin is used orally as well as parenterally for the treatment of urinary tract infections only. Intravenous use of nitrofurantoin does not produce higher urine levels than oral administration or intramuscular injection does[284]. A comparison of all three modes of application has proved that orally-administered nitrofurantoin is absorbed completely and metabolized rapidly since maximum drug levels appear in the urine within thirty to sixty minutes after administration. To reduce the rate of side effects, enteric coated tablets may be administered. Numerous reports especially from Japan[285–288] have led to the conclusion that such tablets are absorbed at a slower rate and are, therefore, probably better tolerated. On the other hand, it seems likely that adequate bacteriostatic levels cannot be achieved and hence a higher failure rate will ensue with this method.

Nitrofurantoin seems to produce a number of untoward reactions with which the physician should be familiar. The most frequently encountered side effects are nausea and vomiting: there are reports which quote an incidence of 33 per cent[289], although the average range will be from 10 to 15 per cent[290,291]. In comparison to these side effects, allergic reactions and drug fever are rather uncommon. Luebbers[292] observed two patients with nitrofurantoin-induced fever and simultaneous shock-like reactions. In both cases the side effects occurred after a single nitrofurantoin tablet; however there had been previous treatment with the same drug. Another patient experienced an anaphylactic reaction with severe dyspnea, wheezing and generalized itching after a dose of 100 mg of nitrofurantoin[293].

Allergic pulmonary infiltration with accompanying eosinophilia also belong to this category[294–296]. A very rare case was reported by Oswald[297] in the Netherlands who noticed a toxic epidermal necrolysis in a 60 year old female who had received nitrofurantoin for 15 days. These signs partially subsided during the next five days. Another rare observation[298] was made on a 7 month old infant who had received crushed nitrofurantoin tablets in four divided doses for 7 days (8 mg/kg daily). Thereafter the drug was continued prophylactically for 60 days (2 mg/kg twice a day). When the baby was 14 months, it was noted that its upper incisor teeth and two lower incisor teeth were stained yellow. Nitrofurantoin should therefore be administered as a pediatric suspension, not as crushed tablets, during tooth eruption. The purpose of this recommendation is to minimize contact with dentition and thus the possibility of discoloration. It is doubtful, however, whether this measure alone prevents the complication. Studies by Strauss and Jawetz[299] eliminated hepatoxicity from the list of side effects. Even in the presence of hepatic dysfunction, nitrofurantoin can be administered[300].

In contrast to other side effects, peripheral neuropathy has only been observed in the presence of 'predisposing factors', namely diabetes mellitus, anaemia, B-avitaminosis, or whenever electrolyte balance was disturbed. It may also occur if the NPN (serum nonprotein nitrogen) is over 100 mg per

cent so that proper elimination of the drug cannot ensue. It was calculated that up to 1962 there had been four cases of peripheral neuropathy in one million nitrofurantoin treatment courses[301,302]. By the end of 1964, another 12 reports were published which described neurotoxic symptoms of various degrees in 25 patients[303–314]. It is noteworthy that neither the duration of treatment nor the ingested amount of nitrofurantoin followed a definite pattern. Sometimes patients developed paresthesias after a few days of treatment. The aforementioned predisposing factors, however, were invariably present. There were various degrees of severity ranging from slight paresthesia to irreversible motor paralysis. The administration of Vitamin B preparations does not seem to be of very much avail in improving symptoms of side effects. Since it is difficult to make recommendations on the prevention of peripheral neuropathy, the suggestion[303] that a daily dose of 400 mg should not be exceeded and that the drug ordinarily should not be given for longer than a 2 week period may be of some value. A smaller dose probably could be given safely for a longer period[303]. This reaffirms the theory that maximum dosage, prolonged therapy, and impaired renal function enhance the likelihood of neuropathy. When considering the many millions of treatment courses during the past 3 years, it seems that the incidence of peripheral neuropathy has decreased rather than increased, which may in part be ascribed to stricter observance of the contraindications with the use of nitrofurantoin.

In primaquine-sensitive individuals haemolytic anaemia may occur. There have been four reports on this side effect since 1962[315–318]. The daily dose varied between 200 and 800 mg of nitrofurantoin and the duration of treatment ranged from three to fourteen days. In all patients, erythrocyte glucose-6-phosphate dehydrogenase values were reduced. Glutathione blood levels were also found to be depressed[316,318]. It must be assumed, however, that this side effect, which occurs only in the Negro, is extremely rare and is also reversible.

De Veber and Valentine[319] described a 2 month old infant with a history of anaemia who was also found to have a urinary tract infection. Besides iron therapy, the baby received 5 mg of nitrofurantoin three times daily. During the following three weeks the child lost weight, became listless, pale and irritable, so that nitrofurantoin was discontinued. At that time the haemoglobin level was 5·6 gm/100 ml. and the reticulocyte count 3 per cent. A subsequent bone marrow examination showed evidence of erythroid hyperplasia and megaloblastic alterations which responded well to folic acid therapy. Another patient with a history of two previous episodes of anaemia developed megaloblastic anaemia 2 weeks after a third course of nitrofurantoin[320]. Clinical pathological studies revealed a folic acid deficiency. Anaemia responded well to the administration of folic acid. Darcus[321] however found no significant changes in ten patients with urinary tract infections who received 200 mg of nitrofurantoin daily for eight days, even in patients with evidence of folic acid deficiency prior to therapy. Peaston[322] also believes that his observation (a gastrointestinal malabsorption syndrome) contradicts the hypothesis which claims that megaloblastic anaemia caused by folic acid deficiency is attributable to the administration of nitrofurantoin. There have been no other observations of this kind in the literature, and we

are entitled on the basis of the available evidence to conclude that megaloblastic anaemia due to folic acid deficiency is not caused by nitrofurantoin.

Another blood dyscrasia which was attributed to nitrofurantoin was described by Chamberlin[323]. He observed a 17 year old male with infectious mononucleosis who had received nitrofurantoin. The etiology was not clearly established, however, and it must be remembered in this connection that bleeding phenomena have been found in 7 per cent of all cases of mononucleosis[324] so that this platelet defect might be considered a complication of the disease rather than a drug effect. Although nitrofurantoin has most often been employed in urinary tract infections, the drug has occasionally been used for the treatment of infantile diarrhoea[325]. It is likely that this is a suitable indication for nitrofurantoin since the amount of nitrofurantoin which passes the intestines may suffice for antibacterial action. When blood and tissue levels, however, are required, nitrofurantoin would definitely not be the drug of choice. This also applies to the use of nitrofurantoin sodium with which Alessandri[326] reported excellent results while treating patients who had undergone pleuropulmonary surgery in tuberculosis.

Nitrofurantoin has been administered rectally[327] in high dosages (10–20 mg/kg) whereby bacteriostatic urine levels were demonstrated. On the other hand, such great amounts of nitrofurantoin may cause rectal irritation and discomfort. For these reasons the manufacture of nitrofurantoin suppositories has largely been discontinued. None the less, patients with urethritis benefit greatly from nitrofurantoin suppositories administered once daily for periods ranging from a few weeks to several months[328]. While in acute, uncomplicated infections 100 mg of nitrofurantoin three times daily will usually suffice, 100 mg four times daily is recommended when there is a history of recurrent infection. Failure of response after seven days of treatment renders a determination of the antibacterial sensitivity of the organisms mandatory. Since the *in vitro* antibacterial activity of the drug is not significantly affected by variation of pH between 5·5 and 8·0, adjustment during nitrofurantoin treatment will not be necessary[329].

For long-term, continuous treatment the lowest effective dose will have to be determined. (It usually ranges from 50–150 mg daily in adults: children receive 2–3 mg/kg body weight.) The drug should be given for at least three days after the urine has become sterile. Administration with meals and/or milk may help to prevent nausea. Since nitrofurantoin does not provide close contact with functional parts, it should be given in combination with a broad-spectrum antibiotic during the early stages of a urinary tract infection. Chloramphenicol appears to be most suitable for this purpose, as its urinary excretion is not affected by reduced renal function. Nitrofurantoin, on the other hand, is poorly excreted in patients with renal insufficiency; therefore its application is contra-indicated whenever the NPN of the serum exceeds 100 mg per cent. Elevation up to 100 mg per cent limits the use of the drug to 50 mg twice daily[330].

Nitrofurantoin does not develop cross-resistance or cross-sensitization with antibiotics or sulphonamides. Super-infection due to *Candida albicans*, commonly encountered with the use of antibiotics, has not been observed with its use. When intravenous nitrofurantoin sodium is administered, 100 to 180 mg may be given twice daily if the patient weighs over 55 kg. Other-

wise, the recommended dose is approximately 6 mg/kg of body weight per day divided into two equal doses. Given over a period of 3 hours, the infusion is well tolerated; given in less than 3 hours, the incidence of side reactions will increase[331]. In contrast to antabuse-like reactions which occur with furazolidone and furaltadone, nitrofurantoin does not have this type of side reaction. There have been no clinical reports of antabuse effects although nitrofurantoin has been used in a great number of patients over many years when it may reasonably have been assumed that some patients did ingest alcoholic beverages during the course of treatment. Experimentally this observation has been confirmed in tests carried out in healthy volunteers[332].

Furazolidone

Subsequent to nitrofurantoin, furazolidone was next to gain worldwide recognition. Like its predecessor which was the first nitrofuran administered perorally to human beings, this product is also ingested by mouth. The clinical indications comprise a great variety of gastro-intestinal infections of different etiology.

Publications on furazolidone during the past three years report the use of this compound in dysentery, typhoid fever and paratyphoid, coli enteritis, giardiasis, brucellosis, trichomonal vaginitis and enteritis of undetermined origin. There is no doubt that for most of these conditions furazolidone has been a useful chemotherapeutic agent. The highest cure rate of all types of infections can probably be achieved in bacillary dysentery caused by the *Shigella* species. A 6 year survey of 329 children[333] indicates that furazolidone (15 mg/kg) had become the drug of choice in these patients (paediatric Shigellosis). There was more rapid clinical improvement with furazolidone than with tetracycline derivatives, sulphadiazine, chloramphenicol or neomycin. Another extensive study[334] on 621 children yielded similarly favourable results, namely a cure rate of 94 per cent was achieved. The failure to obtain a bacteriological cure was ascribed to decreased effectiveness which stood in direct relation to the period of time which elapsed between the onset of the disease and the initiation of treatment. Usually treatment has to be extended over a period of 4 to 7 days. Under exceptionally poor sanitary conditions two weeks may be required[335]. Other publications on this particular indication[336–339] report equally good results, a rather high rate of bacteriological cures and the superiority to sulphonamides and antibiotics in the treatment of Shigellosis.

A great number of papers[340–367] originating mainly in Latin America describe furazolidone as a highly effective agent in the treatment of giardiasis which is a parasitic disease caused by *Giardia lamblia* (the disease is also called lambliasis). During the first seven days of treatment a cure rate of 80–90 per cent[364] can usually be expected. The daily dose for children was 5–15 mg/kg per day on the average, while adults received 100 mg four times daily. In a subsequent bacteriological examination[351], the cure rate was 100 per cent when the post-treatment observation period was extended to ten days.

In coli enteritis which is a rather frequent intestinal infection in infants, furazolidone is also highly effective. Most reports[368–376] which were published during the last three years indicate a cure rate of 75–100 per cent. The daily

dose was 10–15 mg/kg per day. This amount was usually given for a period of 10 days. A large number of cases was observed in Germany from 1960–1962[375]. The summary of the results of 361 courses of treatment indicated that neither fungal nor staphylococcal super-infections were encountered. Side effects in this particular study mentioned vomiting and allergic skin reactions which were termed 'harmless' and occurred in only one per cent of the cases. Salmonella infections also responded to furazolidone, although the cure rate varied considerably, depending on the virulence of each strain (50–90–95 per cent)[375,377,378].

Numerous publications[379–395] report good results in the treatment of infectious gastroenteritis of various etiology. Stool cultures were usually not carried out in this group; the cure rate which fluctuated between 75 and 100 per cent, therefore denotes a 'clinical cure'. Children usually received 10–15 mg/kg daily for a week to ten days, whereas adults were given 300 to 500 mg of furazolidone daily. When comparing the clinical effectiveness of nitrofurantoin and furazolidone[396] a surprising observation was made: in the nitrofurantoin-treated group there was a cure rate of 80 per cent while only 33 per cent were cured in the furazolidone-treated group (12 mg/kg/day for six days). Causative organisms were not identified. It seems, nevertheless, that sufficient nitrofurantoin passes the intestinal tract to affect the bacteria.

Rare indications for furazolidone include its use for the treatment of brucellosis[397]. The cure rate, however, was low (50 per cent) whereas those of chloramphenicol plus streptomycin as well as oxytetracycline plus streptomycin was 85 and 88 per cent respectively. Osha[398] reported good results in the treatment of trichomonal infections in women. A mixture of furazolidone plus nifuroxime plus stilbesterol was used later for this indication under the trade name of Tricofuron. A somewhat different combination also containing furazolidone and diethylstilbesterol was administered in another group of 40 patients[399] for a period of five to eight days. No relapses had occurred after 6 months.

Side effects with furazolidone are usually harmless and consist of slight headache, nausea, occasional vomiting and allergic skin reactions. This drug, however, can produce rather strong antabuse-like reactions[400–402], manifested in facial flushing, headache, nausea, fatigue, thirst and even dyspnea and wheezing. Since such reactions can occur up to 48 hours after the last dose of the drug has been ingested, it is recommended to abstain from alcoholic beverages during the course of treatment and for at least two days after the last dose has been administered.

One of the chief advantages of furazolidone is that this compound is very often effective against strains that have become resistant to antibiotics and sulphonamides. As with nitrofurantoin, there has been virtually no development of bacterial resistance during the past years. The compound with its exceptionally broad antibacterial spectrum affects nearly all organisms causing bacterial diarrhoea. Similar to nitrofurantoin, this compound may cause intravascular haemolysis in certain groups of patients of Mediterranean and Near Eastern origin, as well as in Negroes. This side effect, however, is probably due to an intrinsic defect of red blood cell metabolism which is found in a small percentage of the population of these areas. Numerous other compounds rendered the red blood cells of these individuals particularly

susceptible. Most recently, however, reports of haemolytic anaemia caused by furazolidone cannot be found in the literature.

Furazolidone and nifuroxime

A mixture of 5 mg furazolidone and 7·5 mg nifuroxime in a water-soluble base of polyethylene glycol, polyoxyethylene palmitate and lactic acid is used as a topical agent for the treatment of *Trichomonas vaginalis* and *Candida albicans* infections. The product is available as a suppository or as a powder for topical treatment of these types of infections. If the suppositories are employed regularly, such as in the morning and at night during the first week and only at night thereafter for at least one menstrual cycle and several additional days, a bacteriological cure rate of 75–97 per cent can be expected[403–407] (these figures apply to trichomonas, moniliasis as well as to 'mixed' infections).

Although these earlier reports have been confirmed by more recent observations with similar bacteriological and clinical cure rates[408–410], the value of this mixture has been diminished somewhat by a simpler mode of treatment, namely, the oral management of these conditions. Nevertheless the prime advantage of topical therapy is that infections are treated on the site. More specifically this mixture of furazolidone and nifuroxime not only eradicates the causative agents but also acidifies the secretion and therefore helps to restore the normal vaginal flora. Oral compounds, however, are more convenient to administer and their effectiveness seems to be unchallenged. It is impossible to predict at this point which mode of treatment will be preferred in the future.

Other nitrofurans

In addition to the three major nitrofurans, nitrofurazone, nitrofurantoin and furazolidone, which have gained world-wide recognition during the past twenty years, a few investigational compounds have recently proved successful in the treatment of various conditions.

Since 1963 the Norwich Pharmacal Company have carried out extensive laboratory and clinical trials with 1-[(5-nitrofurfurylidene)amino]imidazolidin-2-one (NF-246, *LXXV*). This product is more effective in *Aerobacter aerogenes*, *Proteus vulgaris* and/or *mirabilis* and *Pseudomonas aeruginosa* urinary tract infections than nitrofurantoin (*III*)[411].

The formulae show that the only structural difference between these two compounds is the replacement of the oxygen at the C_4 position of the hydantoin ring of nitrofurantoin with two hydrogen atoms or perhaps more precisely, the replacement of the hydantoin ring by an imidazolidinone ring. In several strains of *E. coli* and *Proteus*, NF-246 showed greater effectiveness than nitrofurantoin (*Table 6.28*).

These results demonstrate that both nitrofurantoin and NF-246 are capable of controlling the infection. The 10 per cent advantage of NF-246 over nitrofurantoin may or may not be significant.

In Germany, Boehringer & Soehne[95] have developed several new nitrofurans, two of which have been studied extensively. 6-Amino-1-[(5-nitro-2-furyl)vinyl]pyridazine (NF-1002, nifurprazine, p. 354, *LXXIV*) also proved effective against *Pseudomonas aeruginosa* and *Clostridium perfringens*, besides

Table 6.28. Comparison of antibacterial action of nitrofurantoin and NF-246

Species	*Strain*	MIC* (mg%)		*Bacterial species*	*Strain*	MIC* (mg%)	
		NF-246	*nitro-furantoin*			NF-246	*nitro-furantoin*
E. coli	Es-2	0·1	0·3	*Aerobacter aerogenes*	Ae-1	0·3	5
	Es-31	0·07	1·25		Ae-4	2·5	20
	Es-33	0·3	1·25		Ae-6	1·25	10
	Es-36	0·07	1·25		Ae-10	0·15	2·5
	Es-38	0·15	1·25		Ae-12	0·3	5
	Es-39	0·15	1·25		Ae-13	0·6	5
Proteus vulgaris	Pr-12	10	20	*Pseudomonas aeruginosa*	Ps-10	20	20
	Pr-52	10	20		Ps-38	20	20
Proteus mirabilis	Pr-53	10	20		Ps-41	20	20
	Pr-54	20	20		Ps-42	20	20
	Pr-55	10	20		Ps-44	20	20
	Pr-56	10	20		Ps-45	20	20

Also in experimental *E. coli* pyelonephritis in rats the compound was found to be superior:

Drug†	*number of kidneys positive/ total number examined*	*kidneys positive* (%)
Nitrofurantoin	6/20	30
NF-246	4/20	20
Control	19/20	95

* MIC: minimum inhibitory concentration.

† Compounds administered orally at 10 mg/kg twice daily for 2 days.

being one hundred times as effective as nitrofurazone against a wide variety of gram positive and gram negative organisms. A summarizing report[412] indicated that a high clinical cure rate of 95 per cent was achieved with this topical agent. Sensitivity reactions were rare and occurred only in eight out of 2,000 cases, i.e. 0·4 per cent. Nifurprazine is effective in low concentrations against 114 strains out of 32 bacterial species[413]. Especially remarkable is its efficacy against *Clostridia*; and, in comparison with other nitrofurans, its high activity against *Pseudomonas aeruginosa.* Minimum inhibitory concentrations (MIC) of nifurprazine which are effective against some bacterial species commonly found in wounds are given in *Table 6.29.*

Table 6.29. Antibacterial activity of nifurprazine

Species	*Number of strains*	*MIC of nifurprazine* (μg/ml.)
Staphylococcus aureus	10	0·031–0·25
Streptococcus pyogenes	10	0·016–0·25
Escherichia coli	10	0·008–0·062
Pseudomonas aeruginosa	10	2–16
Proteus vulgaris	6	0·125–4

Nifurprazine has a bactericidal action against micro-organisms in the logarithmic growth phase as well as in the stationary phase[414,415]. A 50 per cent content of human serum in the culture medium does not interfere with the bactericidal activity of the compound. Further clinical investigations[416] showed that the compound is very effective against wound infections and a preparation for topical application was marketed in 1966.

The other nitrofuran by the same research laboratories is 1,2-amino-5-[2-(5-nitro-2-furylvinyl)]-2-(5-amino-2-(1,3,4-thiadiazolyl)ethylene (*LX*)[98]. Extensive toxicity studies and excellent *in vitro* results made the clinical usefulness of this compound look promising. During the course of investigation, however, a number of untoward side effects was observed which promptly led to the discontinuation of all studies. A total of 105 urological patients had received this product as a chemotherapeutic agent against urinary tract infections[417]. Initially 500 mg were given and the bacteriological cure rate at this dosage level was only 25 per cent; subsequent therapy was carried out with 1,000 mg per day. Urinary excretion which was checked simultaneously was sufficient to produce adequate bacteriostatic levels. Before studies with this compound could be concluded, however, clinical investigation had to be discontinued because of the following side effects which were observed in 23 patients: eight instances of antabuse-like reactions, seven instances of nausea, three instances of peripheral neuritis, two instances of vomiting, one instance each of fever, anorexia and gastric discomfort. The major concern was the three cases of toxic effects on the peripheral nervous system. All these patients complained of paresthesias in the legs and of limited ability to walk. All symptoms disappeared as soon as the drug was discontinued. In Japan tests with this compound for the treatment of intestinal infections are in progress but final results are not yet available.

A number of reports from Italy[418–431] concern 2-acetamido-4-(5-nitro-2-furyl)thiazole ('Furium'). This compound exhibits a high degree of activity against bacilli and pathogenic enterobacteria. Complete growth inhibition at drug dilution of 4 to 6 μg/ml. was found for *Staph. aureus*, *Sal. typhimurium* and *E. coli*[418]. Although 2-acetamido-4-(5-nitro-2-furyl)thiazole has been used in a number of indications, the best response was observed in cases of cystitis and urinary calculi with secondary infection[420]. In surgery it was used after bile duct operations at a dosage of 400 mg per day. Eleven of the 38 patients, however, reported nausea and gastric discomfort[419]. The drug also seemed to be effective in bacterial enteritis[428]. In this indication 250 to 400 mg per day were given for three to nine days.

Another compound used in Italy is a hydroxymethyl-nitrofurantoin which produces higher urinary concentrations than nitrofurantoin and is also excreted more rapidly[432,433]. When 200 to 300 mg were given orally in divided dosages daily for two to ten days, good results were obtained in 73·5 per cent of the cases[434]. Comparative studies with nitrofurantoin yielded good results in 75 per cent of cases. This compound is also known as Z-56.

2-Amino-5-(5-nitro-2-furyl)-1,3,4-thiadiazole has been used in gastroenteritis (25 mg/kg per day) with 85 per cent efficacy[435], and also as a topical agent for the symptomatic relief of chronic inflammatory disorders of the anorectal area (haemorrhoidal, anal fissures and proctitis)[436,437].

The isopropyl and ethoxycarbonylmethyl derivatives of 3-(5-nitro-2-furyl)acrylamide proved to be effective in the treatment of *Schistosoma japonicum*[438–440]. After one to two weeks of therapy with a total dosage of 700 to 1,500 mg of one of the drugs, 50 per cent of all treated patients had negative stool specimens. The rate of worm elimination in animals (drug doses were not stated) was extremely high: in mice 100 per cent, in rabbits 97 to 100 per cent, and in dogs 85 to 90 per cent[439].

Panfuran acetate or acetylpanfuran, 3-acetylamino-6-(5-nitro-2-furyl-vinyl)-1,2,4-triazine[441] (*Table 6.9*), has been used for the treatment of acute tonsillitis. Although rapid clinical improvement was observed it must be remembered that results are difficult to assess. In the same study, black discoloration of the tongue was noted. Panfuran acetate has also been employed as an ointment[442]. When used in concentrations of 0·03 per cent and 0·5 per cent, this formulation did not seem to cause any irritation (relevant to this point is that its precursor guanofuracin had caused leukoderma of the eyelids and therefore had to be withdrawn despite its good clinical efficacy).

After unsuccessful trials with Panfuran H (3-amino-6-(5-nitrofurylvinyl)-1,2,4-triazine hydrochloride) and panfuran acetate (*Table 6.9*), another compound which was called Panfuran S (3-di(hydroxymethyl)amino-6-(5-nitrofurylvinyl)-1,2,4-triazine) (*Table 6.9*) was synthesized[443]. The compound exhibited good clinical effects in patients with bacillary dysentery. All stool cultures became negative a few days after treatment had been initiated and relapses were not observed. The drug also seems to be free of side effects. Given at different dosage levels for periods of five days (8, 16 and 32 mg/kg), the highest dosage proved the most effective, yielding a bacteriological cure rate of 86 per cent. When employed in urinary tract infections caused by strains which are resistant to antibiotics and sulpha drugs, the drug seems to be rather ineffective in that a bacteriological cure could not be achieved[444]. In this study 1,000 mg per day were given. Side effects (vomiting, epigastric discomfort, anorexia, and diarrhoea) were noted in over 25 per cent of the patients. A publication on the use of Panfuran S in acute pneumonia[445] covers only 5 cases, and is, therefore, not substantial enough to evaluate Panfuran S for this particular indication. Panfuran S demonstrates its greatest efficacy in the treatment of bacillary dysentery although it has also proved to be of some value in other indications; so far, the published material is too scanty to provide conclusive evidence in this respect.

Apart from their antibacterial action the nitrofurans have been tested as antiprotozoic, antihelminthic, antihypertensive and antineoplastic agents[446]. The trials for these indications, however, are still in their neophyte stages, and it remains the task of future researchers to determine the importance of these additional indications for the nitrofurans.

ADDENDUM

The following is a selection of papers which have appeared since the main part of this review was completed. Further variants of the azomethine type of compound (p. 322) have been prepared, for example, those of general formula 5-O_2N·Fur·CH=NR in which R is a 5-substituted 3-rhodanine[447], 3-quinazol-4-one[448], 2- or 6-acetamidonicotinamido[448] or 4,6-disubstituted 2-pyrimidinylamino[449]. Vinylogous nitrofurans (p. 329) continue to attract attention; the general formula 5-O_2N·Fur·CH=CHR has been varied by synthesizing compounds in which R is 2-imidazoline[450], 2-(1,3-oxazole)[451], 2-(4-alkylthiazole *N*-oxide)[452], 2-(1,3,4-oxadiazole)[453], 2-(1,3,4-oxadiazolin-5-one)[454], 4-substituted 2-(1,3,4-oxadiazolin-5-one)[454,455] and 2-quinolin-4-ol[456]. Some vinylogues that carry a methyl group attached to the vinyl group have been studied[457,458].

Considerable effort has also been directed recently to nitrofurans in which a heterocyclic ring system is directly attached to C_5 of the nitrofuran (p. 333). A compound of this type possessing broad-spectrum antibacterial activity has been described by workers[459] from the laboratory which discovered the usefulness of this type of compound[112]. In this new compound, the 5-nitrofuryl ring is attached to a 2-(3-pyridyl)thiazole at C_4 of the thiazole. Slight variations of this structure have been investigated[460]. This nitrofuran, like a few others[447,449,450], also possesses antitrichomonal action. Other heterorings which have been directly attached to the nitrofuran include 6-(2-alkylpyridazin-3-one)[461], substituted 2-benzimidazole[462], 2-(4-aminoquinazoline)[463], 5-(3-alkyl-1,2,4-triazole)[464] and 3-imidazo[2,1-b]thiazole[465]. Many of these papers give detailed information on the antibacterial activity of the compounds and a Russian paper[466] discusses the effect on potency of simple variations in the furan ring. When 5-nitrofurfuraldehyde is condensed with polyacryloyl hydrazide, a polymeric nitrofuran is obtained[467], which has a greater duration of action than do other nitrofurans. It is not absorbed when given orally to rats, and after a single parenteral dose, the urine has antibacterial properties for a much longer time than with nitrofurantoin. A *p*-phenylene homologue of an acrylamide has been synthesized[468].

A new spectroscopic method of determining nitrofurantoin in the urine is claimed to distinguish it from any of its metabolites[469] and from a number of antibiotics[470]; the same method may be used to determine nitrofurantoin in whole blood or plasma[471]. Polarographic and microbiological methods of determination have been applied[472] to a mixture of nitrofurantoin and NF-246 (pp. 354 and 365). Some aspects of the metabolism of nitrofurantoin have been studied; for example, plasma binding has been estimated[473] to be 60 per cent. Under conditions of acid urine, about 30 per cent of the total amount of filtered and secreted nitrofurantoin followed non-ionic back-diffusion. Theoretically, this amount may be sufficient for a chemotherapeutic effect in renal interstitial tissue. A comparison of normal and impaired renal function on the excretion of nitrofurantoin given intramuscularly or orally has been investigated[474]. Excretion was prolonged by renal insufficiency but administration of probenecid to a number of patients significantly reduced the rate of excretion. Administration of nitrofurantoin

intravenously, intratracheally or by inhalation as an aerosol gave high concentrations of the drug in the urine[475]. Absorption and distribution studies[476] on NF-246 (pp. 354 and 365) in the dog showed that the compound is well absorbed when given orally.

Numerous clinical studies continue to show the superiority of nitrofurans; for example, in the treatment of urinary tract infection[477] in 1,100 patients, nitrofurantoin gave good to very good results compared with sulphonamides and antibiotics. The sensitivity figures of urinary tract organisms for 1959, 1963 and 1966 showed no change to nitrofurantoin[478]. The usual route of administering nitrofurantoin is peroral, but recently[479] intraperitoneal injections in normal saline were successful in combating infections (especially staphylococcal) of the abdominal cavities. Pregnant women with urinary infection responded favourably[480] to a daily dose of nitrofurantoin throughout pregnancy and for 8 weeks post-partum.

Furazolidone inhibits monoamine oxidase, and as its effect is cumulative, prolonged treatment with the drug may induce susceptibility to hypotensive episodes from exogenous amines such as tyramine[481]. The drug has been withdrawn in Germany for this reason.

Nitrofurfuryl methyl ether and nifuroxime (p. 323) are effective antifungal agents[482]; diacetoxymethylfuratriazine has given good results in the treatment of dysentery[483]; and finally, Nidrafur (5-nitro-2-furaldehyde acetylhydrazone) protected turkeys from histamoniasis more effectively than Antobax (4,7-phenanthroline-5,6-quinone)[484].

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