PROGRESS IN MEDICINAL CHEMISTRY 16

G. P. ELLIS
G. B. WEST
EDITORS

Progress in Medicinal Chemistry 16

This Page Intentionally Left Blank

Progress in Medicinal Chemistry 16

Edited by
G.P. ELLIS, D.SC., PH.D., F.R.I.C.
Department of Chemistry,
University of Wales Institute of Science and Technology,
King Edward VII Avenue,
Cardiff, CF1 3NU

and

G.B. WEST, B.PHARM., D.SC., PH.D., F.I.BIOL.
Department of Paramedical Sciences,
North East London Polytechnic,
Romford Road,
London E15 4LZ

1979

NORTH-HOLLAND PUBLISHING COMPANY
AMSTERDAM·NEW YORK·OXFORD

ISBN for the series: 0 7204 7400 0
ISBN for this volume: 0 7204 0667 6

PUBLISHERS:

Elsevier North-Holland Biomedical Press
335 Jan van Galenstraat, P.O. Box 211
Amsterdam, The Netherlands

SOLE DISTRIBUTORS FOR THE U.S.A. AND CANADA:

Elsevier/North-Holland Inc.
52 Vanderbilt Avenue
New York, N.Y. 10017, U.S.A.

LIBRARY OF CONGRESS CATALOGING IN PUBLICATION DATA

Main entry under title:

Progress in medicinal chemistry.
London, Butterworths, 1961–1973 (Vols. 1–9).
Amsterdam, North-Holland Publishing Co., 1974– (Vols. 10–).
Editors: 1961– G.P. Ellis and G.B. West.
Includes bibliography.
1. Pharmacology–Collected works. 2. Chemistry, Medical and Pharmaceutical. I. Ellis, Gwynn Pennant, ed. II. West, Geoffrey Buckle, 1961– ed. III. Title: Medicinal chemistry.
RS402.P78 615'.19 62–2712

Photosetting by Thomson Press (India) Limited, New Delhi
Printed in The Netherlands

Preface

We have pleasure in presenting five reviews in this volume. Recent trends towards international conformity in patent law are now beginning to show material benefits. The significance of these changes for medicinal chemists is outlined in Chapter 1. Steroids containing hetero atoms have been extensively studied in recent years and their synthesis and biological activity are described in Chapter 2, which is a longer review than is usual in this Series. The fight against neoplastic diseases continues to be waged on many fronts and one of these — with drugs which exhibit non-bonded interactions with nucleic acids — is surveyed in Chapter 3. The role of membranes in chemotherapy is well-established and Chapter 4 covers the inhibition by drugs of adenosine triphosphatase from microbial membranes. Finally, Chapter 5 assesses the progress made recently in our understanding of neuromuscular blockade at the cholinergic receptor.

Much effort is required to prepare reviews of this type and we are indebted to our authors for their diligence and punctuality. Owners of copyright material have freely given their permission for it to be reproduced in this volume and we are grateful to them. As usual, the staff of our publishers have given us their full co-operation and it is a pleasure to acknowledge their contribution.

November 1978

G.P. Ellis
G.B. West

This Page Intentionally Left Blank

Contents

This Page Intentionally Left Blank

Contents of earlier volumes

VOLUME 8

VOLUME 9

VOLUME 10

VOLUME 11

VOLUME 12

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

VOLUME 13

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

VOLUME 14

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

VOLUME 15

1 THE USE OF STABLE ISOTOPES IN MEDICINAL CHEMISTRY—D. Halliday
2 MEMBRANE-ACTIVE ANTIMICROBIAL AGENTS—P.A. Lambert
3 ADRIAMYCIN AND RELATED ANTHRACYCLINE ANTIBIOTICS—J.R. Brown
4 THE HYPOPHYSIOTROPHIC HORMONES—J.C. Buckingham
5 COPPER COMPLEXES—A UNIQUE CLASS OF ANTI-ARTHRITIC DRUGS—R.J.R. Sorenson
6 NON-TRICYCLIC ANTIDEPRESSANTS—J. van Dijk, J. Hartog and F.C. Hillen
7 THE CHEMISTRY .AND BIOLOGICAL ACTIVITY OF α-(N)-HETEROCYCLIC CARBOXALDEHYDE THIOSEMICARBAZONES—K.C. Agarwal and A.C. Sartorelli
8 PROSTAGLANDINS AND THROMBOXANES—M.P.L. Caton and K. Crowshaw

Progress in Medicinal Chemistry — Vol. 16, edited by G.P. Ellis and G.B. West

1 Recent Changes in Patent Law

F. MURPHY, B.Sc., M.R.I.C., C.P.A., E.P.A.

Fisons Ltd., Ipswich, Suffolk IP1 1QH, England

INTRODUCTION

A number of major developments in the comparatively calm world of patents occurred in 1978. These included a new Patents Act in the United Kingdom,

the commencement of operation of the European Patent Office and the institution of operations under the Patent Cooperation Treaty. Additionally, in February 1978 an important decision of the Constitutional Court in Italy nullified Article 14 of the Italian Patents Act, which had prohibited the grant of patents for pharmaceuticals. These various developments have or will have a significant effect on the procedure for obtaining patents and the importance of patents both generally and particularly as regards pharmaceuticals.

It is probably well understood that a patent is the means by which monopoly rights are granted for inventions. A brief history is given in an earlier survey [1]. The first of the modern Patents Acts was that of 1907 which introduced examination of patent applications for the first time, thus placing the United Kingdom in the group of countries that followed the philosophy of examination of patents before grant. This Act prohibited the claiming of chemical compounds per se, provided assistance to the developing British chemical industry which was at that time overshadowed by its German counterpart, and additionally provided for the grant of compulsory licences in respect of patents for food and medicine. The 1907 Act, partially amended by the Acts of 1919, 1928, 1932, 1938 and 1942, effectively remained in force until it was repealed and replaced by the Patents Act 1949. One of the most important changes introduced by the 1949 Act was that, in response to pressures from the then well developed British chemical industry, chemical compounds per se could be claimed, thus reverting to the situation which existed prior to the 1907 Act.

The protection of chemicals generally and of drugs in particular has always been sensitive to politically related pressures, on the one hand from industry, and on the other hand from the public interest. There appears to be a fairly widespread belief in the theory that a strong patent system is of benefit in a country with a developed research based industry but may hinder a country with a developing industry; additionally there is the suspicion—largely unjustified—that the protection secured by patents for new drugs leads to or induces higher prices for such drugs. Nevertheless, a properly organised patent system is advantageous to all countries.

Many complex factors are involved in the assessment of the effect of patents on chemical industry generally and on the pharmaceutical industry in particular. The general conclusion from objective investigation is that the patent system encourages and stimulates invention and innovative development which leads to new chemical products and new drugs of benefit to the community at large.

In a sense, part of the difficulties in terms of the chemical industry and more particularly the pharmaceutical industry has been that at the time of the main

development of theory and practice regarding patent laws, developments in industry were mainly in the mechanical and engineering fields, and the provisions of the law were designed to deal with such inventions. As already noted, discrimination against chemical and particularly pharmaceutical inventions are to be observed both historically and territorially, although the rational view in terms of patent law is that there should be no discrimination between different types of invention.

Patents are just one part, albeit an important part, of the protective elements existing in the field of industrial property, which includes patents, designs, trade marks, copyright and knowhow, and needs to be considered in this overall context, as described in an earlier survey [1]. In many fields, and certainly in respect of drugs, there is a significant time-related connection between patents and trade marks, since the patent is of paramount importance in the earlier years of the life of a drug whereas the trade mark becomes of paramount importance in later years, after patent expiry.

THE PURPOSE OF THE PATENT SYSTEM

The generally recognised purpose of the patent monopoly system is to encourage the development of science and technology and to assist in the dissemination of new inventions. The conclusions of the Banks Committee which examined the operation of the patent system in the United Kingdom in the late 60's [2] were that patents systems played an important role in encouraging innovation, that no alternative system had emerged and that national patent systems were of importance in worldwide development of technology. The essential characteristics of the patent monopoly system are, on the one hand, publication, and on the other hand a monopoly for a determined period of years. The time at which publication occurs and the term of the monopoly are factors which have been changed both in the United Kingdom and several other countries as a result of recent legislation.

In any patent system, three factors are of primary significance:

(1) the date of publication of the patent;
(2) whether or not there is examination of the patent before grant, and
(3) the duration of the patent (patent term).

It is an essential feature of all patent systems that the patentee is required to make a full disclosure of the invention, so that the invention can be carried out by third parties. The time of publication is normally either at a fixed time after

filing or priority date (usually 18 months after the priority date) or after the examination has been completed—at acceptance or grant.

EXAMINATION OF PATENT APPLICATIONS

The question of examination is one point on which there has always been the biggest philosophical difference between patent systems. On the one hand, there were those countries, *e.g.* United Kingdom, Germany, U.S.A., Japan, which believed that patents should only be granted after examination, so that industry should not be burdened by the existence of granted patents which clearly were invalid; the examination system thus provided a screening operation which either removed at least the most invalid patents or resulted in a patent grant which had at least presumptive validity. On the other hand, there were those countries, *e.g.* France, Belgium, Italy, Spain, which took the view that in any litigation the validity of the patent would be challenged anyway and preliminary examination was really a duplication of effort; therefore, the grant procedure by the Patent Office should merely be a bureaucratic exercise with examination restricted to purely formal aspects, and the patent, when granted, could then be attacked or ignored by third parties as they wished. The latter philosophy, although it may appear pragmatic, has lost favour essentially for the reason that the grant of clearly invalid patents is vexatious and troublesome to industry as a whole; consequently there has been a considerable swing in favour of a pre-grant examination procedure. Nevertheless, so far as the 'examination countries' are concerned, because of a combination of factors including complexity of technology and diminishing resources in the Patent Offices, an intolerable backlog in examining patent applications built up. As a result, the concept of belated examination was introduced under which a patent would only be granted after examination, but the applicants could delay for a number of years, *e.g.* 7 years, the request for the examination process. This device certainly reduced the pressures on the Patent Office since, on the one hand, it spread the load over a longer period, and on the other hand those cases of minor or transient importance would never come up for examination because during the 7 year period these would be dropped by the applicant.

A feature of the belated examination arrangement was that the applications would be published at a fixed early date, *i.e.* prior to examination, normally 18 months after the priority date. Belated examination is not regarded as an ideal solution and indeed may be regarded as encompassing the disadvantages of the two previous systems without any real compensating advantage. Certainly

the three new systems introduced in 1978, although these include the concept of early publication, have not included the concept of belated examination.

TERRITORIAL JURISDICTION

The grant of a patent is restricted to the territorial limits of the Government giving the grant; thus, a British patent gives a monopoly only within the territory of the United Kingdom. Although the new systems which came into effect in June 1978 as the result of international collaboration still lead to individual national patents, nevertheless these point the way to a wider system. Even so, it could not be said that we are within sight of a universal patent, although we are probably within sight of a Common Market patent. From the drafts of the Common Market Patent Convention which have been issued so far, it is clear that under this Convention, a single application will lead to a single grant effective for the whole of the Common Market. Some aspects still remain to be resolved, such as whether the Common Market Patent when granted is divisible or indivisible and what legal systems will be used for the determination of infringement, but these issues will be resolved and the Common Market Patent should come into being within the next 2—3 years.

INTERNATIONAL CO-OPERATION ON PATENT LAW

Although the United Kingdom Patents Act 1977 will be discussed specifically, the inter-relation of the European Patent Convention, the Patent Co-operation Treaty and the Patents Act needs to be understood. To some extent, the key to this legislation is the European Patent Convention which was signed by 16 states at a Diplomatic Conference in Munich in October 1973 (*Table 1.1*).

Table 1.1. SIGNATORIES OF THE EUROPEAN PATENT CONVENTION

Austria	Liechtenstein
Belgium	Luxembourg
Denmark	Monaco
France	Netherlands
Germany	Norway
Greece	Sweden
Ireland	Switzerland
Italy	United Kingdom

Additional countries which may later accede to the European Patent Convention are Cyprus, Finland, Iceland, Portugal, Spain, Turkey and Yugoslavia. The terms of the European Patent Convention provided the basis upon which the European Patent Office would be set up and the basis upon which patents would be granted by this Office. The European Patent Convention provides a system for a single filing, a single search, a single examination, a single grant process and a single opposition (limited in time to 9 months after grant) which leads not to a single patent but to a bundle of national patents which are thereafter enforced nationally in the individual countries concerned. A necessary corollary to the single examination process leading to the grant of a bundle of national patents is that the European Patent Convention and the national patent systems have to correspond. What has therefore been happening as a gradual process since 1976 has been that the various signatory states have been amending their Patents Acts to correspond more or less to the European Patent Convention. This Convention came into force on the 7th October 1977, when seven of the signatory states, namely Germany, Netherlands, United Kingdom, France, Belgium, Luxembourg and Switzerland ratified the Convention. Subsequently Sweden has ratified the Convention. The national Patents Acts, e.g. the British, needed amendment to conform with the characteristics of early publication (18 months from the priority date), searching and examination before grant (but not on a belated examination basis), and a patent term of 20 years running from the date of application. It will be seen from this that the concensus view of most countries is that examination before grant coupled with early publication is the best system and that a patent term of 20 years is a reasonable figure.

The Patent Cooperation Treaty is an internationally collaborated system for filing patent applications with a broader territorial basis than Europe, and

Table 1.2. COUNTRIES RATIFYING THE PATENT COOPERATION TREATY

Brazil	Luxembourg
Cameroon	Madagascar
Central African Empire	Malawi
Chad	Senegal
Congo	Sweden
Denmark	Switzerland
France	Togo
Gabon	United Kingdom
Germany	U.S.A.
Japan	U.S.S.R.

it predates the European Patent Convention since the Treaty was signed in Washington in June 1970, although it did not come into operation until the 1st June 1978. Under this arrangement, by a single application an effective filing for countries within the Patent Cooperation Treaty may be made which is followed by a single search; thereafter examination is dealt with on a national basis, leading after national examination to the grant of national patents. Twenty countries have ratified the Treaty to date (October 1978) (*Table 1.2*).

UNITED KINGDOM PATENTS ACT 1977

The changes to the Patents Act 1977 bring the Act into conformity with the European Patent Convention, and as a result the provisions of the new Act are more representative of patent law in other countries than was the case with the 1949 Act.

PATENTABLE INVENTIONS

The 1977 Patents Act defines positively a patentable invention as one (a) in which the invention is new, (b) which involve an inventive step, (c) which is capable of industrial application, and (d) which is not excluded by the specific exceptions.

The specific exceptions are:

(a) a discovery, scientific theory or mathematical method;
(b) a literary, dramatic, musical or artistic work or any other aesthetic creation whatsoever;
(c) a scheme, rule or method for performing a mental act, playing a game or doing business, or a program for a computer;
(d) the presentation of information;
(e) inventions which would encourage offensive, immoral or anti-social behaviour;
(f) any variations of animal or plant or any essentially biological process for the production of animals or plants, not being a micro-biological process or the product of such a process.

The old tests on patentability regarding what was a manner of manufacture are now superceded and the essential characteristic is that the invention is capable of industrial application. Nevertheless the criteria pronounced in 1943 by Mr Justice Morton (as he then was), [3] that a process was patentable if it '(a) results in the production of some vendible products, or (b) improves or

restores to its former condition a vendible product, or (c) has the effect of preserving from deterioration some vendible product to which it is applied' are still largely applicable, although the current definition may possibly be wider in some respects although possibly not in others.

Essentially all processes and products of an industrial character are patentable. Thus any new chemical process is patentable, and so is any new chemical compound. Furthermore, any new composition, be it a pharmaceutical composition or any other type of composition, is also patentable. There are new provisions in part extending and in part limiting the scope of patentable inventions; the notable limitation is in relation to veterinary inventions. Industrial application means that the invention is capable of being made or used in any kind of industry including agriculture; but this definition includes the rider that any method of treatment of the human or animal body by surgery or therapy or method of diagnosis practised on the human or animal body should not be construed as an industrial application. What this means in practice in terms of inventions in the veterinary and agricultural area is that while agrochemical processes related to the treatment of land crops are clearly patentable, processes involved with animal husbandry, although this is clearly a part of agriculture, come into an area of doubt. It would seem that there is a dividing line between the individual treatment of animals in response to a diagnosis, which now seems to be not patentable, and routine treatment not in response to diagnosis, such as for example indiscriminate treatment with drugs to secure uniform growth or the inclusion of drugs as a food supplement which is patentable. The precise position of that dividing line will, no doubt, be determined in a series of contested cases.

Of particular interest to the pharmaceutical industry is the fact that biological fermentation processes are clearly patentable, and special provisions are made for the deposit of new cultures in named culture collections. The problem has been that where a new micro-organism is required for a new process — for example, to produce a new antibiotic — the sufficiency requirements for validity (which essentially mean that the public must be able to carry out the invention from the published description in the patent) are opposed to the reasonable requirement of the inventors that the micro-organism does not become freely available before he has secured enforceable patent rights. The rules under the new Act, which correspond also to the European Patent Convention requirements, provide that the sufficiency requirement is met if

(a) a culture of the micro-organism is deposited in a culture collection not later than the date of filing of the application,
(b) the application gives the available characteristics of the micro-organism, and

(c) the name of the culture collection, the deposit date and number are given in the specification.

The making of such deposit is taken as the irrevocable consent of the inventory to the culture being made available to third parties after publication of the application subject to such parties obtaining certificate of release from the Comptroller of Patents on the basis of

(1) undertaking not to make the culture available to any other person before the patent expires, and
(2) undertaking to use the culture only for experimental purposes prior to patent grant.

These provisions safeguard the rights of the inventor, while permitting bona fide research. Where the culture has to be exported, and such export is prohibited by law, the inventor is required to make a further deposit in a country from which the culture would be available to parties obtaining the certificate of release.

One important extension of what is patentable relates to the discovery of new uses of known materials, and the new Act provides that a known substance or composition for use in the method of treatment of the human or animal body by surgery or therpay or for diagnosis shall be patentable providing such known substances or composition has not previously been described as suitable for that use. It is interesting to note that this provision corresponds to a similar provision in the European Patent Convention, but in the latter Convention a pharmaceutical use of a known compound or composition is only patentable if this is the first known pharmaceutical use for that known compound or composition.

In connection with any invention in the pharmaceutical field, it is possible to protect the medicament itself where this comprises either a new chemical compound or a new composition, and also to protect methods for the production of the pharmaceutical compound where such methods are novel. Where the end product is novel, the process is deemed to be novel, even though the method may be known as a general method applicable to similar compounds. This is the so-called 'Analogy Process', which is of particular significance in countries which do not permit chemical compound cover. Up to this point, there is no real difference between the protection of a chemical invention and a pharmaceutical invention, and where the invention is for a new compound or composition the resulting protection is fully effective. The difference between chemical inventions and pharmaceutical inventions emerge in terms of use since in the chemical field generally inventions directed to methods of use are patentable where in the pharmaceutical field inventions

directed to methods of use are not normally patentable. The claims mentioned above for compositions for use in a method of treatment are not 'method of use' claims but use-bound composition claims. Even where a compound or composition is novel, the additional protection derived from claims directed to use may be desirable and in some cases important. However, where the compound or composition is known, the only protection possible is that derived from use-bound composition claims, and these new provisions in the U.K. Patents Act are certainly welcome.

The exclusion from protection of any method of treatment of the human or animal body creates a limit to the area of patentable inventions where the dividing line between patentable and unpatentable may be susceptible to some movement. Thus, for example, a method of contraception has been found to be patentable under the old Act, and possibly will be found to be patentable under the new Act on the basis that this does not comprise a method of treatment by surgery or therapy. Similarly, under the old Act methods for the treatment of hair were found to be patentable, for the interesting reason that a woman's hair was considered to be a vendible article — in the words of the decision 'which is not only capable of being sold on the head, but has been so sold frequently abroad and less frequently in this country [4]. It would seem that under the new Act a method for the treatment of the hair, to the extent that this could not be regarded as a method of therapy, would be patentable. Similarly, it would appear that under the new Act, methods for the treatment of the body for purely cosmetic purposes which can be demonstrated as not being a method of therapy, should also be patentable.

In most countries an invention to be patentable must relate to an industrial process, although the definition of what is meant by an industrial process varies somewhat from country to country. However, the European Patent Convention has provided a standard definition, which is largely followed in the new UK Patent Act, and which is being followed in many other countries.

NOVELTY AND INVENTIVENESS

For an invention to be patentable, in addition to being capable of industrial application, the invention must also be new. The new UK Patents Act introduced an important change in the definition of novelty, this change corresponding also to the definition in the European Patent Convention. Under the old Act, an invention was novel if this had not been published in the United Kingdom — this is the concept of local novelty, which philosophically is related to innovation in the industrial development in the country concerned, and reflected the slow transfer of knowledge and technology in

times past. One aspect was that if someone developed for the first time in the United Kingdom an invention which had not previously been known in the United Kingdom, this represented an important industrial development in this country and thus entitled the inventor to a patent. Another aspect reflecting the need to encourage industrial development was that travellers abroad who then brought the technology back to the United Kingdom were regarded as meritorious. Thus under the old Act patents were granted to persons who had obtained inventions overseas, following the practice established in the Clothworkers of Ipswich case in 1615, which provided that anyone who brought a new invention or commenced a new trade in the United Kingdom should be granted a patent 'because at first the people of the Kingdom are ignorant and have not the knowledge and skill to use it' [5]. However, these concepts are now rather out-of-date, particularly in view of instantaneous world-wide communications as we know it today, and the new Patents Act provides for absolute novelty, by which is meant that an invention to be novel at the priority date shall not be available to the public anywhere in the world by written or orally published description, by use or in any other way. The novelty requirement is dramatically widened compared with the old Act since novelty is not only destroyed by printed publications before the priority date in any part of the world, but is also destroyed by oral description or use anywhere in the world. What is meant by 'made available to the public by oral description', still remains to be determined precisely; however it certainly does not apply to the private conversation of two individuals. On the other hand, it clearly does apply to oral disclosure at any sort of meeting to which the public have a right of access, be this scientific or non-scientific. Where the dividing line lies between a public meeting and a private discussion is still to be determined. It is, however, an interesting facet of the new Act that a British Patent application may now be anticipated by what is said in a public meeting in Timbuctu. Equally, novelty can be destroyed by use of the invention anywhere in the world. However, such prior oral descriptions or use would have to be capable of clear proof before these could be effective to destroy novelty of a patent application.

Another new feature introduced into the Patents Act 1977 resolves the relationship between co-pending applications. In the past there have been two main theories of how to deal with this situation. The first was the 'prior claim theory' which effectively meant that where there were two co-pending applications, the first patentee was entitled to claim whatever he was entitled to on the basis of his application, and then the second patentee was only able to claim what was not specifically claimed by the first patentee providing this

was properly based on the second application. The other approach is known as the 'whole contents approach', and the basis of this is that as between the two co-pending applications, the earlier application ought to be treated as a prior publication for the purpose of determining what claims could be allowed in the later application. The old Act followed the prior claim basis; the new Act follows the whole contents approach. Thus, where there are two co-pending applications (i.e. the later application was filed before the earlier application was published), for the purpose of determining novelty of the later application the earlier application is treated as a publication; however the later application cannot be refused on the basis that the invention is obvious over the content of the earlier application. In other words, the later application has only to pass the test of strict novelty compared with the content of the earlier application.

To be patentable, an invention must be new and involve an inventive step; to involve an inventive step, the invention must not be obvious to a person skilled in the art, having regard to what is known. What is meant by obvious has not really changed under the new Act. Many decisions bear on this question, and to a large extent whether or not an invention is obvious depends on the attitude of those skilled in the art to which the invention relates. The standard to pass the test of obviousness in the United Kingdom has been rising over the years—for example there were cases some years ago where the application of techniques well known in one industry to another industry was not considered to be obvious. Equally, however, there is a fairly general recognition that many inventions appear obvious, once they have been made, although the problem they solve has previously eluded solution. As a result, the measurement of obviousness after the event can present difficulties and where the identification of a solution to the problem has eluded people working in the field for some time, this clearly establishes that the solution, although it may appear obvious once propounded, was in fact not obvious.

PROCEDURE FOR FILING

The new Act has changed and to some extent simplified the procedure for filing patent applications, one of the main differences being that there is no longer a provisional specification and a complete specification. However, an equivalent procedure exists inasmuch as it is possible to file an informal application followed by a formal application. An informal application is filed by a single patent form — Patents Form No. 1/77, called 'Request for the Grant of a Patent', which includes the title, the name of the applicant, and the name of the inventor, and is followed by the specification. The specification

should effectively correspond to the old type provisional specification and include a description of the invention, how the invention is carried out, and suitably include examples. The current cost of filing an informal application is £5 (compared with the cost of £1 for the former provisional specification). To complete such an application so as to arrive at a formal application, two alternative steps may be taken. The first is merely to file claims to be attached to the specification together with the additional formal requirements, namely appropriate drawings, if any, an abstract, the request for the search and a statement of inventorship and right to apply. The fees involved for converting the informal to a formal application amount to £40. The alternative method is to file a further application within 12 months from the date of the original application claiming priority from the first application, this later application being in the form of a formal application including not only a complete description but also the claims, the abstract, the request for search, and the statement of inventorship and right to apply. The costs involved in this case amount to £5 plus the supplementary costs noted above, namely £45 in all.

Of the two alternatives, the latter, although it costs £5 more, is the preferred course, since the term of the patent is 20 years from the date of the patent, and in the former case the cate of the patent will be the date of the informal application whereas in the latter case the date of the patent will be the date of the second and formal application. There is no requirement for the inventor to sign any document, unless the inventor is an applicant, and even as regards the application, there is no need for the applicant himself to sign any document if he has appointed an agent, since the appointed agent may sign all the documents. Obviously, however, if the applicant so choses, he need not start the process by filing an informal application, but could file a formal application immediately, together with all the necessary requirements, but this will involve the corresponding shortening of the patent term.

The first filing, be this formal or informal, will provide the basis for priority, not only as regards the United Kingdom application, but as regards any application filed overseas claiming priority, which is filed within 12 months of the original filing date. Consequently, the content of the description in the original filing needs to be as comprehensive and as detailed as possible, since if there are any deficiencies, and someone else is working in the same area, the other applicant may end up with the better priority date. The 'whole contents' approach for co-pending applications, means that the fuller description is of benefit in defeating a later application, and makes it more difficult for the later applicant to show novelty over the earlier application. However, although such full and comprehensive disclosure is of particular benefit in overcoming the threat of patent applications from third parties, it should be recognised

that this is not without hazard for the applicant himself, since the whole contents approach to co-pending applications applies not only as between co-pending applications of different applicants, but also co-pending applications of the same applicant. Where two such applications are filed and dated within one year of each other, this should not present a problem, since the two applications could be cognated (*i.e.* combined) together, in effect forming two priority dates for the same formal application. However, where the two applications are dated more than 12 months apart, this would not be possible, and the later application would need to be novel over the disclosure in the earlier application. This situation is referred to as 'self collision' and means that the scope of an application needs to be carefully considered, weighing up the risks of others working in the same area against the possibility of development of ancillary aspects of the invention. Every case needs to be considered on its merits, but as a general rule it would seem better to attempt to achieve full disclosure of the invention as understood and claimed, but only to include fringe areas if these clearly are not likely to be the subject of further development.

For all countries of the world with the exception of the United States of America and Canada, one of the most important features of the patent is its date, since in any conflict situation, the patent goes to the first to file. By contrast with this, in the United States of America and Canada, within the limits of the particular legislation discussed later, the patent goes to the first to invent.

The relationship between the informal application and the formal application is merely one of priority entitlement; where the invention claimed in the formal application is supported by matter disclosed in an earlier application from which priority is claimed, the priority date of that invention is such earlier filing date. Priority can be claimed from one or more earlier applications, and such earlier applications need not only be United Kingdom applications, but can be applications in any Convention country. However, for priority to be claimed, all the relevant applications have to be filed or at least stand in the name of the same applicant. Priority, however, can only be claimed in an application filed within 12 months from the earlier priority date claimed. Applications filed under the new Act will be published automatically 18 months after the priority date. Thus, in a case where a formal application is filed without any priority, this will be published 18 months later; in the case where an informal application is filed and a formal application is filed within the following 12 months, publication will take place within 18 months of the filing of the informal application. In other words, publication takes place 18 months after the earliest priority date declared. This publication can only be

prevented if the applicant withdraws the application prior to publication, or will only be delayed where the applicant withdraws or cancels the claim to priority. It appears that with the bureaucratic processes, unless some action is taken 16 months from the priority date, the application will inevitably proceed to publication at the 18 months date. This will be the first publication of the application, and publication will be with a seven serial number commencing with 2 million preceded by the letter A. The second publication will take place after grant and will have the same serial number preceded by the letter B.

EXAMINATION

Almost immediately after the formal application is filed, a preliminary examination and search of the application will be made by the Patent Office. Under the old Act, the Patent Office only had to consider British Patent specifications and literarure references restricted to those published within 50 years before the priority date. Under the new Act, the Patent Office have to search for novelty as such within the meaning of the Act, namely the search should embrace the world patent and technical literature without time limit. Obviously the Patent Office will not immediately be able to switch from a restricted search to a wide search, but this change will be gradually undertaken. The preliminary examination made at this stage is merely a formal examination to ensure that the application complies with the formal requirements, which will include whether or not more than one invention is involved. Unity of invention is generally related to the question of the search and the Patent Office, in the past, have only been prepared to make one search per application. The result of the formal examination and search is communicated to the applicant who, possibly in the light of the search result, may decide not to proceed. Substantive examination of the application has to be requested (and the corresponding fee paid) within 6 months of the publication of the application or 2 years after the declared priority date. On substantive examination the Examiner will consider the application as a whole and the search results. In addition to the editorial function which traditionally has been one of the functions of the Patent Office Examiner, under the new Act the Examiner is entitled to consider not only novelty but also obviousness. The British Examiner, therefore in the substantive examination will be in an equivalent position to that of the United States and German Examiners for many years past. Just how the British Examiner will react to this new found power of refusal remains to be seen, but it is expected that at least in the foreseeable future that the attitudes and practices adopted in the British

Patent Office will be as reasonable and rational as they have been in the past. Following completion of the examination, and presuming that the Examiner has been satisfied that the invention is patentable, the application passes to grant and is printed for the second time, but as noted above, with the original number prefixed by the letter B.

REVOCATION

Another change made in the Patents Act 1977 is that opposition within three months of publication no longer applies, and indeed opposition as such prior to grant no longer exists. In place of this, at any time during the life of the patent an interested party can apply to the Patent Office for revocation of the patent on any of five grounds, which are the same grounds as in the European Patent Convention. These five grounds are:

(a) the invention is not patentable;
(b) the patent was granted to a person not entitled to the grant;
(c) the specification does not disclose the invention clearly and completely enough for it to be performed by a person skilled in the art;
(d) the matter in the patent goes beyond the disclosure as filed by the applicant;
(e) the protection secured by the patent has been extended during examination in a way which should not have been allowed.

These grounds consolidate some of the old grounds of opposition and revocation; however, some new grounds are added — (d) and (e) above — and some of the old grounds are removed — namely false suggestion and not a manner of manufacture.

In general the most frequent attack on any patent, at any stage, has always been, and is likely to be in the future, on the general ground of patentability, be this on lack of novelty or obviousness. The first ground of revocation in the new Patents Act certainly covers lack of novelty plus obviousness; however it also includes miscellaneous grounds such as that the invention is not capable of industrial application — *i.e.* is not an invention. Furthermore where the situation involves two co-pending applications, patentability in the light of the 'whole contents' philosophy also falls under this heading.

The second ground relating to grant to the wrong person is also not really new, and exists to enable any person who has been deprived of rights by the patentee to secure some form of restitution. The loss of rights could arise either through devolution of title or because the invention was obtained from the claimant.

The third ground corresponds to the old ground of insufficiency, and goes to the basic concept of the patent grant, which is that the monopoly is based on a full disclosure of the invention being made by the patentee. Although insufficiency has been quite a frequent ground of attack in opposition and revocation proceedings, it is not a ground which has been notable for its success, and this will probably also be the case in the future. The fourth and fifth grounds are both new and related to one another. The fourth ground is primarily directed to amendments made during prosecution, *e.g.*, where divisional applications are filed, where perhaps the specification includes new matter, and the fifth ground relates to amendments made to the patent, presumably as a result of an amendment procedure under section 76 of the Act. Although these last two grounds tidy up matters so far as amendment is concerned, they are unlikely to be of major significance in revocation proceedings since one would have presumed that amendment or extensions of the type referred to would not have been permitted during the examination processes.

FEES

The Government fees involved in patent applications are probably still modest but viewed in the context of the fees applying in 1962 are very substantial indeed. The cost of filing an informal application is £5, the request for preliminary examination costs £40, and the request for substantive examination costs a further £40. No fee is required to secure grant of the patent so that apart from supplemental fees due to claiming the priority date etc, the total minimum cost to grant is £85. Renewal fees start with the fifth year and are shown in *Table 1.3.*

Table 1.3. RENEWAL FEES OF PATENTS IN THE UNITED KINGDOM

5th year	£40	13th year	£ 84
6th year	£42	14th year	£ 92
7th year	£46	15th year	£100
8th year	£50	16th year	£108
9th year	£56	17th year	£118
10th year	£62	18th year	£128
11th year	£68	19th year	£140
12th year	£76	20th year	£152

OTHER IMPORTANT CHANGES

It is no longer possible to invalidate a patent on the ground of prior use. In place of this former attack on validity, the prior user has the right to continue doing whatever he was doing before the date of the patent. This provision, sometimes referred to as a shop right, corresponds to the European Patent Convention, and the situation in other countries, *e.g.*, Germany. The right to continue applies to a party who in good faith either carries out the invention or makes 'effective and serious preparation' to do so, before the priority date of the claim which would otherwise be infringed.

The definition of infringement has been expanded so as to cover 'contributory infringement'. In other words, the offer or sale of a product for use in an infringing way or process, where the seller knows or ought to know that the product will be so used, comprises an infringement. These provisions do not apply to staple commercial products (*e.g.*, standard chemicals) unless the sale or offer was to induce the purchaser to infringe. Thus, for example, where a patent covers the use of a compound for a method of treatment, there is infringement where third parties sell that compound associated with instructions relating to the method of treatment.

Possibly the most revolutional changes in the Patents Act bear on the rights of inventors. The new provisions apply to inventions made after the 1st June 1978, and have two effects:

(a) a statutory definition is provided for the rights in inventions made by employees;
(b) provisions are made for awards for inventors.

The definition of the rights of employers and employees corresponds very closely with the Common Law position before the Act. Prior to the Act, the terms of any contract between the employee and employer prevailed over Common Law — unless the contract was unreasonable. Under the new Act, the definition in the Act supercedes any contract between the employer and employee. The definition of rights is as follows:

(1) Notwithstanding anything in any rule of law, an invention made by an employee shall, as between him and his employer, be taken to belong to his employer for the purposes of this Act and all other purposes if

(a) it was made in the course of the normal duties of the employee or in the course of duties falling outside his normal duties, but specifically assigned to him, and the circumstances in either case were such that an invention might reasonably be expected to result from the carrying out of his duties; or

(b) the invention was made in the course of the duties of the employee and, at the time of making the invention, because of the nature of his duties and the particular responsibilities arising from the nature of his duties he had a special obligation to further the interests of the employer's undertaking.

(2) Any other invention made by an employee shall, as between him and his employer, be taken for those purposes to belong to the employee.

One effect of the new Act is that in respect of an invention where the rights belong to the employer, the invention is taken 'to belong to his employer for the purposes of this Act and all other purposes'. In other words, the employee no longer holds the rights in trust, and has these rights to assign to the employer, as was previously the case, but has no rights at all since these already belong to the employer. Consequently, for UK purposes, inventors no longer have to execute assignments, although these are still required overseas.

The most dramatic change concerns inventions made after 1st June 1978, and the new Law provides that in certain circumstances an award of compensation may be made to employees who are the inventors in patents owned by their employers, which patents have been commercialised; different conditions govern the awards made for inventions belonging to the employer and for inventions belonging to the employee.

In the case of inventions belonging to and patented by the employer, an award of compensation will be made where the patent (having regard among other things to the size and nature of the employer's undertaking) is of outstanding benefit to the employer.

The award of compensation will be such as to secure for the employee a fair share of the benefit derived by the employer having regard to

(a) the nature of the employee's duties, his remuneration and the other advantages he derives or has derived from his employment or has derived in relation to the invention under this Act;
(b) the effort and skill which the employee has devoted to making the invention;
(c) the effort and skill which any other person has devoted to making the invention jointly with the employee concerned, and the advice and other assistance contributed by any other employee who is not a joint inventor of the invention, and
(d) the contribution made by the employer to the making, developing, and working of the invention by the provision of advice, facilities and other assistance, by the provision of opportunities and by his managerial and commercial skill and activities.

In the case of inventions made in the pharmaceutical area in industrial research organisations, it seems clear that many years must elapse before the first patent of outstanding benefit will emerge. It seems probable that having regard to all the factors, the award is unlikely to be very substantial. Additionally, under present tax laws, any such award would be taxed as income.

In the case of patented inventions belonging to the employee and patented by the employer (*i.e.* on the basis of an assignment from the employee to the employer) an award for compensation may be made when the benefit secured by the employee is inadequate in relation to the benefit derived by the employer from the patent. Inventions in this category are rather uncommon in the pharmaceutical field, but where they occur, it will produce more for the inventor, who additionally would probably be able to secure payment without tax deduction.

PATENTS IN OTHER COUNTRIES

In view of the harmonisation of laws following the Patent Cooperation Treaty and the European Patent Convention, the definition of patentability in many countries corresponds generally with that of the new UK Patents Act. Thus, for example, France, Germany, Switzerland, Denmark and the United Kingdom have modified the Patent Law to correspond with the EPC or PCT. The main exception in relation to pharmaceutical inventions are those countries which have discriminatory laws or regulations. These include prohibition of patents for pharmaceuticals (*e.g.* Brazil and formerly Italy), limitation of patents to process cover (*e.g.* Canada), limitation of patent type (*e.g.* Mexico) and limitation of patent term (*e.g.* India and Costa Rica). This discrimination extends to chemicals generally, and a number of countries still do not permit claims to chemicals *per se*, irrespective of their use. The main industrial countries, however, conform generally with the new Conventions.

The determination of novelty and inventiveness is, however, still subject to quite significant differences. Firstly, there is the issue as to whether the invention has only to be new in the individual country (local novelty) or whether the invention has to be new in view of publications anywhere in the world (world novelty). Secondly, there is the question of obviousness, and the standard of inventiveness to be applied. In Germany and Holland, in addition to novelty and obviousness in a general sense, there has also been the requirement of 'inventive height', by which is meant that some unexpected result or feature is required for patentability.

To a large extent, in the past, the test for obviousness in the United Kingdom was the easiest and the test in Holland the most difficult, with Germany not far behind. The European Patent Convention which uses essentially the same words as the British Patent Act now embraces all the systems and the key question for the European Patent is what level of obviousness is going to be required to establish patentability. The Guidelines provided by the European Patent Office recognise the problem and the solution propounded is that the test should be higher than in the United Kingdom lower than in Germany.

The United States of America has the broadest definition of what comprises an invention, and the definition reads as follows:

> 'whoever invents or discovers any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof, may obtain a patent therefor'.

This definition covers essentially all inventions, including medical and pharmaceutical processes. Thus the definition does not include the limitation of relating to an industrial process, and unlike the situation in the European Patent Convention, any process for the treatment of the body by surgery or therapy is patentable.

In permitting the patenting of medical processes, the U.S.A. stands almost alone, except for this having shared with a small and diminishing group of countries including Belgium, Philippines, South Africa and Australia. However, in South Africa, medical processes will become unpatentable on the 1st January 1979, and in Australia, although the Patent Office, as a result of administrative procedures, grants patents for medical processes, the decisions of the Court bearing on this are to the effect that such processes, if these are directed to a method of curing a disease, ameliorating a malfunction or rectifying a disorder, are unpatentable. U.S.A. has subscribed to the PCT, but is not entitled to participate in the EPC, although US applicants may file at the EPO. The provisions of US Law have not changed; the patent term remains at 17 years from grant and medical processes as well as chemical products and pharmaceutical products are patentable. The Interference procedures still remain — the principle being that the patent should be granted to the first to invent in the United States of America, as opposed to the first to file as in most other countries. Apart from this, practice is not really so different from that in Europe as regards issues such as novelty and sufficiency of description. The formal requirements for filing have been greatly simplified, and it is no longer necessary for the application documents to be ribboned together or signed before a Notary Public. It is still, however, necessary for the inventor to file the

application, and an assignment is required if the patent is to be granted to the inventor's employers.

In Canada, the law is a blend of UK and US law. Pharmaceutical inventions are discriminated against in that, firstly, only chemical process cover is obtainable and, secondly, that Compulsory Licences are readily available. As in U.S.A., the patent goes to the first to invent, but in Canada the Conflict proceedings are to determine the first party to invent anywhere in the world.

Details of what types of claims may be obtained in some leading countries, and the novelty requirements are given in *Table 1.4.*

Table 1.4. TYPES OF CLAIMS ALLOWABLE AND NOVELTY REQUIREMENTS

Country	*Claims to chemical compounds generally*	*Claims to chemical compounds where a pharmaceutical*	*Claims to pharmaceutical compositions*	*Chemical process cover including pharmaceuticals*	*Novelty*
Argentina	Yes	No	No	Yes	World
Australia	Yes	Yes	Yes	Yes	Local
Austria	No	No	Yes	Yes	World
Belgium	Yes	Yes	Yes	Yes	World
Brazil	No	No	No	No	World
Bulgaria	No	No	No	Yes	Local
Canada	Yes	No	Yes*	Yes	World
Ceylon	Yes	Yes	Yes	Yes	Local
Chile	No	No	Yes	Yes	World
Colombia	No	No	No	No	Local
Czechoslovakia	No	No	Yes	Yes	Local
Denmark	Yes	No	Yes*	Yes	World
Finland	Yes	No	Yes*	Yes	World
France	Yes	Yes	Yes	Yes	World
Germany West	Yes	Yes	Yes	Yes	World
Germany East	No	No	No	Yes	Local
Greece	Yes	No	Yes	Yes	Local
Holland**	Yes	Yes	Yes	Yes	World
Hungary	No	No	No	Yes	Local
India	No	No	No	Yes	Local
Ireland	Yes	Yes	Yes	Yes	World
Israęl	Yes	Yes	Yes	Yes	World
Italy	Yes	Yes	Yes	Yes	World
Japan	Yes	Yes	Yes	Yes	World
Yugoslavia	No	No	No	Yes	Local
Mexico	No	No	No	Yes†	Local
New Zealand	Yes	Yes	Yes	Yes	Local

Table 1 4. (*contd.*)

Country	*Claims to chemical compounds generally*	*Claims to chemical compounds where a pharmaceutical*	*Claims to pharmaceutical compositions*	*Chemical process cover including pharmaceuticals*	*Novelty*
Norway	Yes	No	Yes	Yes	World
Pakistan	Yes	No	Yes	Yes	Local
Paraguay	Yes	No	No	Yes	Local
Peru	Yes	Yes	Yes	Yes	Local
Poland	No	No	No	Yes	Local
Portugal	No	No	Yes	Yes	World
Rumania	No	No	No	Yes	Local
South Africa	Yes	Yes	Yes	Yes	World
Spain	No	No	No	Yes	World
Sweden	Yes	Yes	Yes	Yes	World
Switzerland	Yes	Yes	Yes	Yes	World
Taiwan	No	No	No	Yes	World
Turkey	Yes	No	No	Yes	Local
Uruguay	No	No	No	No	World
U.S.A.	Yes	Yes	Yes	Yes	World
U.S.S.R.	No	No	Yes*	Yes	World

*Pharmaceutical composition should possess some novelty *per se*, *e.g.* a new composition of a known pharmaceutical.

**New law.

†Inventors certificate only.

PATENTABLE INVENTIONS — ITALY

A particularly interesting situation has arisen in Italy, as a result of a decision of the Constitutional Court in February 1978. Article 14 of the Italian Patent law prohibited patent protection for pharmaceuticals, and as is well known, the effect of this prohibition in Italian patent law meant that for a great many years there has been no effective patent protection in Italy for pharmaceuticals. The result has been that a number of Italian companies manufactured in Italy new pharmaceuticals patented in other countries. Despite the existence of Article 14, a number of enterprising patent applicants still filed patent applications in Italy relating to pharmaceuticals, some of which overtly referred to pharmaceutical properties and some of which omitted completely the specific disclosure that the compounds or compositions concerned were pharmaceuticals. The Italian Patent Office routinely rejected such appli-

cations, and in many cases these rejections were the subject of Appeals to the Italian Court of Appeals. Such legal processes in Italy are rather slow-moving, and in 1976 a series of Appeals before the Court of Appeal were referred to the Italian Constitutional Court for a determination as to whether Article 14 of the Patent Law was unconstitutional. With some measure of cooperation, the parties involved in the Appeals presented arguments to the Constitutional Court, and in the end this Court reached the decision in February 1978 that Article 14 of the Patent Law was unconstitutional. With effect from the date of publication of that decision, Article 14 is deemed to be cancelled from the Patent Law, and consequently from that date pharmaceuticals are patentable in Italy. However, before any patent for a pharmaceutical may be granted in Italy, it has to pass through the administrative processes of the Italian Patent Office, which can be time consuming, with the result that although pharmaceuticals have been *de facto* patentable since February 1978, up to the date of writing, it is not believed that any Italian patent specifically covering pharmaceuticals has been granted. The final passage of the Constitutional Court decision reads as follows:

> 'Obviously, it is not the task of this Court to suggest consequential provisions. The legislator must consider whether for a transitional period it is necessary to provide for such inventors who, during the period in which the patent protection was prohibited, did not even file a patent application, so that a situation has been created which in some ways is similar to that of prior use; and whether for a transitional period it is necessary to provide protection for those who have made investments in the pharmaceutical industry, while patent protection could not be obtained, trusting in the consequences deriving from the existence of that legislation.'

Effectively, what this means is that the Constitutional Court were saying that on the one hand those patentees who, in the period up to February 1978, had not filed patent applications in Italy because of Article 14 ought to be provided with some remedy enabling them to rectify the situation, and on the other hand that those Italian manufacturers who quite legitimately, in view of Article 14 of the Patent law, up to February 1978 commenced the manufacture of pharmaceuticals, ought to be protected against the subsequent grant of patents covering their operations. Various proposals for modification of the Italian Patent Law have been made, a characterising feature being the provision of what amounts to a compulsory licence for any Italian manufacturer who commenced operations before February 1978. However, the drafting of acceptable legislation, and particularly the passage of such legislation through Parliament, is a slow process, and it may well be some time

before any modified law comes into effect in Italy. At the present time, the situation is that with the cancellation of Article 14, pharmaceutical inventions are no different from any other chemical invention and full protection for such inventions, as for example in the United Kingdom, is obtainable.

PROCEDURES FOR SECURING PATENTS OVERSEAS

A United Kingdom patent extends only within the United Kingdom; after grant this may be registered in a number of colonial and former colonial territories which are set out in *Table 1.5*. In these registration territories, all that is required is a simple formal application to register the British patent; after registration, the patent is the same as the British patent, and remains in force for the same term as the British patent, without further fees. The patent in the registration territory cannot be attacked; any attack has to be on the basic British patent. The registration system provides a simple patent system for those countries which do not consider it worthwhile to set up separate Patent Offices. In addition, a number of countries with their own Patent Offices and independent filing and examination systems also permit the filing of applications based on foreign granted patents. These are usually termed 'Patents of Confirmation' and the countries where this procedure is followed are also set out in *Table 1.6*.

In all other countries which have a patent system at all, individual patent applications in the appropriate language and form have to be filed in the

Table 1.5. BRITISH REGISTRATION TERRITORIES

Aden	Gambia	Seychelles
Antigua	Gibralter	Sierra Leone
Bahamas	Gilbert and Ellice	Singapore
Bahrain	Islands	Sri Lanka
Barbados	Grenada	St Kitts Nevis and
Belize	Guernsey	Anguila
Bermuda	Guyana	St Helena
British Soloman	Hong Kong	St Lucia
Islands	Jamaica	St Vincent
British Virgin	Jersey	Somaliland
Islands	Kenya	Tanganyika
Brunei	Malaya	Trinidad and Tobago
Cayman Islands	Montserrat	Uganda
Cyprus	Sabah	Western Samoa
Dominica	Sarawak	Zanzibar
Falkland Islands		
Fiji		

Table 1.6. PATENTS OF IMPORTATION, REVALIDATION, CONFIRMATION ETC.

Argentina	Guatemala	Spain
Bahamas	Haiti	Tangier
Belgium	Honduras	Uruguay
Bolivia	Iran	Venezuela
Burundi	Iraq	Zaire
Costa Rica	Panama	
Dominican Republic	Paraguay	
Ecuador	Ruanda	

individual country and are subject to separate examination and grant, with the exception of those countries which have ratified either or both of the two new international arrangements which came into force on the 1st June 1978. One is the European Patent Convention (EPC), and the other is the Patent Cooperation Treaty (PCT). It should be recognised that, as explained in detail below, each of these systems leads ultimately to individual national patents. These systems may loosely be regarded as composite national filings.

Any person resident in the United Kingdom may file a patent application overseas provided either an application for a patent for the same invention has been made in the United Kingdom at least six weeks before the application overseas and no directions as to secrecy have been made, or written permission has been obtained from the Comptroller of the Patent Office. Refusal of permission is unlikely in a patent concerned with drugs, since the provisions are concerned with the prevention of disclosure of information bearing on National Security.

The majority of overseas territories subscribe to the International Convention, which provides that where an application is filed in any one of the Convention countries, an application filed in any other Convention countries within one year of the first date of application is entitled to the date of that first application. Thus, where an application — formal or informal — is filed in the United Kingdom, the applicant has one year in which to decide whether he wishes to secure overseas protection, and if applications are filed within the 12 month period in a convention country, those applications are entitled to the date of the basic British application. Both the EPC and PCT fall within the International Convention, so that a filing in either of these systems can claim the benefit of Convention priority from an earlier national filing; conversely where the first filing is an EPC or PCT filing, this can be used as a basis for Convention priority for national filings.

The provisions regarding the filing requirements vary to some extent from

country to country, but over the past few years there has been considerable harmonisation and the impetus of both the EPC and the PCT has been to introduce even greater conformity. As a result, the form of the patent specification has been largely standardised for most countries; the nature of the claims, however, still varies to some extent from country to country, and advice on the precise form of the claims is the professional responsibility of the attorney handling the application.

EUROPEAN PATENT CONVENTION

The EPC is different from other patent conventions and has involved the setting up of a completely new Patent Office in Munich called the European Patent Office (EPO) with Branch Offices in Berlin and The Hague. The EPO is fully staffed with examiners, administrators and supervisory officials and operates in a similar way to most national Patent Offices. The Branch Office at The Hague is responsible for all searches carried out by the EPO, and was formed from the former International Patent Institute. The Branch Office in Berlin is responsible for the examination of applications in certain classes of subject matter.

The EPO processes patent applications through to the grant of the patents, and thus, in a sense, is a competitor to the individual national Patent Offices. The hope of the EPO is that by rationalisation and standardisation of examination methods, the EPO route to patents will be seen as more efficient and more economical than the national route.

The provisions as regards patentability under the EPC correspond very closely to the definitions in the new British Patents Act, (or to be more precise the British Act conforms to a great extent with the EPC) so that separate detailed consideration is not required. As previously noted, the EPC is a member of the International Convention, so that Convention filings may be made at the EPO. Thus for most British applicants and indeed most applicants, the procedure to be followed will be a national filing — for the British applicant in the United Kingdom — followed within the Convention year by a filing in the EPO. The documents required to file a European Patent Application comprise the Request for the Grant of a European Patent, the Specification, any appropriate drawings, the patent claims and an abstract. The application may be filed in English, French or German, although at a later stage a translation of the claims and/or the abstract and/or the whole specification into the language of the nominated countries may be required. The Request is the effective patent application form which includes the title of the invention, the name and address of the applicant, the name of the

Table 1.7. EUROPEAN PATENT OFFICE FEES

Filing fee	£123
Search fee	£395
Designation fee (per state)	£ 61
Fee for extra claims over 10 claims, per claim	£ 14
Renewal fees to maintain the application	
Third year	£ 90
Fourth year	£120
Fifth year	£150
Sixth year	£184
Seventh year	£218
Eighth year	£266
Ninth year	£313
Tenth year	£382
Examination fee	£470
Grant fee	£ 90
Opposition fee	£123
Appeal fee	£150

inventors, and the name of the representative (if any). The priority claimed also has to be stated, and a designation has to be made as to which of the EPC States where protection is required. The fees are made up of the filing fee, designation fee and the search fee. In the designation of states, it is desirable to put the most important countries first, since if there is a deficiency in the fees, the effect may be that the Patent Office will cancel some of the designated states, and such cancellation would be taken from the end of the list. The basis for the fees is set out in *Table 1.7.*

It will be seen from this that the additional fees for the designation of countries in the EPC is comparatively small. Consequently if there is sufficient interest to file in the EPC, it is probably economical to designate all the contracting states, unless exceptionally it should happen that any one or more of the contracting states are clearly without significance to the particular invention. All the desired countries must be designated at the time of filing and it is impossible subsequently to add to the list of designated states. This is why it is desirable to list all states from the beginning; at a later stage deletions can be made as appropriate or desired.

The EPC may be filed directly at the EPO in Munich or at the Branch Office in The Hague, or at the British Patent Office (and certain other national Patent Offices). For the British applicant, the simplest course of action is to file the papers at the British Patent Office; the filing fees have to be paid after

notification of receipt by the EPO giving the number of the patent application, to the Bank Account of the EPO in London.

Since the EPO has only been operative since June 1978, it has not been administratively possible for all classes of invention to be examined from the beginning. If an application is filed for an invention which is not subject to examination, the European Patent Application has to be converted to a national filing, with the loss of EPO fees. However, so far as pharmaceuticals are concerned, virtually all inventions will be covered by the examination fees and open for examination from the commencement of operation of the Office.

After filing of the application, this will be subject to a preliminary examination as to formal requirements in the Receiving Section of the EPO in Munich, and this formal examination will provide the confirmation of the date of filing, and that the appropriate fees and translation have been filed. Providing that the basic documentation comprising:

(a) the request for a European Patent,
(b) a designation of at least one contracting state,
(c) an identification of the application, and
(d) the specification with at least one claim

is filed, a filing date will be accorded even if there are formal defects and the Receiving Section will notify the applicant and allow a period of one month within which to notify the deficiencies. Once the formal application has been completed, the file is then passed to the Branch Office in The Hague which will carry out the appropriate search. The EPC envisaged that the search would cover all published matter; however, although the search will be of high quality and hopefully all embracing, it is subject to certain economic limitations, so that there will be limits applied to the search at the discretion of the Search Examiner. The search will only be directed to one invention, so that if the Search Examiner considers that more than one invention is involved, the search will only be a partial search directed to one of the inventions, and in such event, the applicant is advised that if the search is to cover the other invention(s), a further search fee must be paid. The payment of additional search fees can be delayed until substantive examination takes place. Such delay seems prudent since the Search Examiner only produces an opinion about unity of invention and the decision on this question rests with the Examining Division in Munich. It should be noted however, that since the issue is essentially a practical one related to the search, the Examining Division is unlikely to differ from the Search Examiner, but by the time of substantive examination the applicant will have a better idea of the importance of the case. When the search is completed, the search report is

communicated to the applicant, is sent to the Examination Division, and is published with the application.

The European Patent Application will be published 18 months from the priority date, which means that in the case of a convention filing only 6 months remains after filing and before publication. It is the intention of the EPO to publish the application and the search report simultaneously; however, with the strict time limits, it seems possible that simultaneous publication may not be obtainable at least in some cases. In such event the application will be published at the 18 month date, without the search report but with the classification attributed by the Search Examiner. The Search Report will be published separately when available. The European Patent application will not be published only if it is either withdrawn by the applicant or refused in sufficient time before the termination of the technical preparations for publication.

If the applicant wishes to proceed after receipt of the search report, he must file a Request for Examination. The time period for filing such Request and paying the appropriate fee, is six months from the date of notification of the publication of the search report in the European Patent Bulletin. The applicant does not have to wait for the search report before requesting substantive examination, but if he does, the Patent Office will ask the applicant to confirm that he still wishes to proceed with the application, after he has received the Search Report.

The application and the search report, and any amendments proposed by the applicant as a result of the search report will be passed to the EPO in Munich for substantive examination. The Substantive Examiner is not expected to search but should check whether there are any conflicting European applications too recent to have appeared in the report from the Search Examiner. The Substantive Examiner examines the application to decide whether or not the claims cover a patentable invention having regard to the search report and other relevant circumstances; inventive height as well as sufficiency of description and the general form of the specification will all be considered. Any one interested in any patent application may write to the EPO with comments on patentability, which have to be considered by the Substantive Examiner.

Objections to the application will be sent to the applicant who has the opportunity to respond and/or amend the claims. It is understood that a time will be set for reply, which will normally be between 2 and 4 months, but in special cases 6 months. The Examiner will consider the response and may either communicate again with the applicant or alternatively pass the file to the Examining Division with a recommendation either to refuse or to grant.

The expectation is that in the majority of cases, the application will be referred to the Examining Division after no more than two communications from the primary Examiner. If the recommendation of the primary Examiner is to allow the patent, it is expected that the application will be allowed automatically; in the event that the Examiner recommends refusal, the application has to be considered by the Examining Division before whom it is possible to have an oral hearing.

If the Examining Division should reject then the applicant has the right of appeal to the Board of Appeal. Since the EPO is new, the nature of the examination can only be the subject of speculation. However, the EPO is made up of Examiners from various national Patent Offices, including both British Patent Office and the German Patent Office, so that the expectation is that when a standard approach is arrived at by the Office, this will represent an examination more difficult than examinations before the British patent Office under the old Act, but less difficult than that in the German Patent Office.

When the application is in order the applicant must approved the text of the patent, pay the grant and printing fees (and renewal fees if any) and file a translation of the claims in the other two official languages. When these formalities are completed, the European Patent is granted and is again published; and the grant is also published in the European Patent Bulletin. The European Patent after grant is open to opposition for 9 months from the date of notification of grant in the European Patent Bulletin. The form of the opposition is similar to that under the new British Patents Act. The only grounds upon which the European Patent may be opposed are:

(a) that the subject matter is not patentable in the sense of being not new or not involving an inventive subject or not susceptible to industrial application
(b) that the patent does not disclose the invention in a manner sufficiently clear and complete for this to be carried out by a person skilled in the art
(c) the subject matter of the European patent extends beyond the content of the application, or
(d) that the subject matter is excluded from patentability, *e.g.* is concerned with scientific theories, computer programs, plant or animal varieties.

It is expected that the essential grounds for opposition will be on lack of novelty or inventive merit, and since this 9 month period represents the only time at which the European Patents are going to be subject to central attack, there is some expectation that a large number of oppositions will be filed. Once the opposition period has passed, the grant procedure results in the grant of individual national patents in all the designated states, which have exactly the

same status as ordinary patents, and are subject to procedures thereafter only on a national basis, either as regards revocation or infringement procedures. Current experience, for example, is that about 30% of German patent applications are opposed; it must be expected that a much higher percentage of European patents are likely to be opposed, and some observers quote figures as high as 90%.

After grant, to secure the individual national patents, the applicant has to file translations (either of the claims of the full specification) and pay fees to the national Patent Offices. Since there may be a substantial period between filing of the application and grant of the patent, the rights of the applicant in this period may be important. Providing that the applicant (where required) files a translation of the claims in the national Patent Office at the date of publication, provisional protection exists from this date in all the designated states in that application. This provisional protection means effectively that after the grant of the patent, the infringer may be sued and be liable for compensation back to the date of publication of the application.

PATENT COOPERATION TREATY

The PCT has not involved a new patent office — although an organisation is involved — but comprises a unified filing and search system for the contracting states. A PCT application can be filed in the Patent Office in any of the contracting states, and generally has to be in one of the official language(s) of that Patent Office. Thus, for example, a PCT filed in London should be in English; similarly a PCT filed at the EPO may be in English, French or German. What is secured under the PCT is an effective filing date in all the designated states with a single application, followed by a single search. The search may be carried out by the Patent Office at which the application is filed, or in another Patent Office. Providing the filing complies with the formal requirements and the appropriate fee (see *Table 1.8* for present fees) is paid, a search is carried out to determine novelty. The search report is sent to the

Table 1.8. PATENT COOPERATION TREATY FEES

Basic fee	£83
Designation fee	£21 per state
Handling fee	£25
Transmittal fee	£ 5
Preliminary examination fee	£25

applicant, and where the applicant wishes to proceed (and pay the necessary fees) the application and the search report are sent to the individual Patent Offices in the countries designated. Thereafter the applications are processed nationally as though they were national applications. The PCT thus provides a simplified filing procedure of assistance to patent applicants who consider the convenience offered is justified by the fees.

CONCLUSION

The changes within Europe have thus led to a greater simplification and uniformity of the patent system which more than counterbalances contrary developments in certain other countries.

REFERENCES

1. F. Murphy, Prog. Med. Chem., 2 (1962), 1.
2. The British Patent System; Report of the Committee to examine the Patent System and Patent Law, HMS 1970, Cmmd 4407.
3. General Electric Co's Application (1943) 60 RPC 1.
4. British Patent No. 552, 285.
5. Godbolt's Reports, K.B. 252.

This Page Intentionally Left Blank

Progress in Medicinal Chemistry — Vol. 16, edited by G.P. Ellis and G.B. West

2 Heterosteroids and Drug Research

HARKISHAN SINGH, M.Pharm., Ph.D.
VIJAY K. KAPOOR, M.Pharm., Ph.D. and
DHARAM PAUL, M.Pharm., Ph.D.

Department of Pharmaceutical Sciences, Panjab University, Chandigarh 160014, India

INTRODUCTION

The steroid system, selected by the evolutionary process to perform some of the most fundamental biological functions, has not only inspired the biochemists and endocrinologists, but has also become the basis of the most phenomenal developments in organic chemistry. The field is of significant interest to medicinal chemists. The broad spectrum of biological activity within the group and the multiplicity of actions displayed by certain individual members make the steroids one of the most intriguing classes of biologically active compounds.

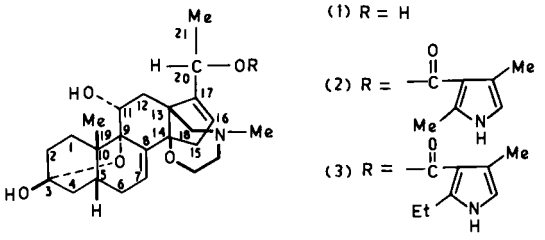
Heterosteroids have recently received much attention. The altered chemical and physical properties are envisaged to lead to the discovery of new substances having the potential of providing some useful drugs. The interesting structural and stereochemical features of the steroid nucleus provide additional attraction to the researcher.

One may divide heterosteroids into nucleo- and extranucleo-categories. The former contain heteroatoms in the nucleus, whereas in the latter, heteroatoms form part of a fused or spiro ring system, an attached group, or a side chain.

HETEROSTEROIDS OF NATURAL ORIGIN

Among the heterosteroids of natural origin, the more prominent are extranuclear azasteroids. Several categories of steroidal alkaloids [1—4] belonging to this group are well-known. These include alkaloids present in *Solanum* [5—8], *Veratrum* [5,8—11], *Holarrhena* [12,13], *Buxus* [11,13], *Sarcococca* [13], *Pachysandra* [13], *Funtumia* [3], *Malouetia* [3], *Kibatalia* and *Paravallaris* [3], *Chonemorpha* [1], and *Fritillaria* [2].

Recently, the steroid alkaloidal components of the Colombian arrow poison frog *Phyllobates aurotaenia* have been investigated [14,15]. The active fraction was separated into batrachotoxin, isobatrachotoxin, and pseudo-

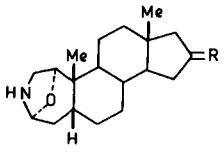


batrachotoxin. The structures of batrachotoxinin A (1) [14], batrachotoxin (2) [15], and homobatrachotoxin (3), the former 'isobatrachotoxin' [14], have been determined. Extensive synthetic studies of structures related to these principles and partial synthesis of batrachotoxinin A (1) have been accomplished [16].

Batrachotoxin is a most active cardiotoxin and has ED_{50} value intravenously in anaesthetized dog of 0.5 $\mu g \cdot kg^{-1}$ [14]. The effects of batrachotoxin in neuromuscular preparations, both pre- and post-synaptically, in nerve axons, in the superior cervical ganglion, in heart Purkinje fibres, and in brain slices appear to be due to the selective and irreversible increase in permeability of membranes to sodium ions [17]. Batrachotoxin therefore is a valuable tool for the study of ion equilibria across membranes [18] and an active interest in the study of its pharmacology continues [19—24].

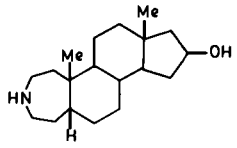
Different nucleoazasteroids have been obtained from the parotid and skin glands of salamanders [3,25,26]. Samandarine (4) is a central nervous system convulsant. Some of the constituents such as samandarone (5) [27], samanine (6) [28,29], cycloneosamandione (7) [30,31] and certain related entities have now been synthesized [32—38].

An interesting group of azasteroids, of which A25822B (8) is an example with high antifungal activity have been isolated from the crude fermentation broth of the fungus *Geotrichum flavo-brunneum* NRRL-3862 [39]. Among

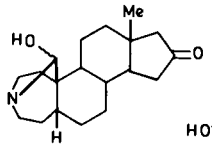


(4) R = 

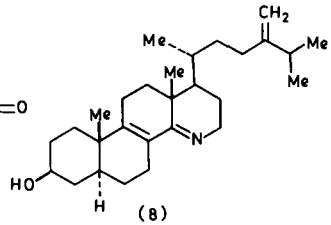
(5) R = O



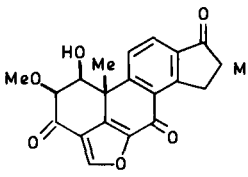
(6)



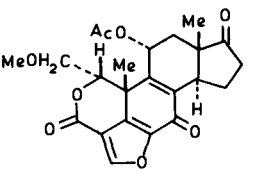
(7)



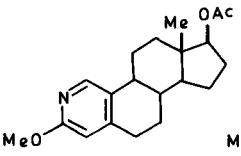
(8)



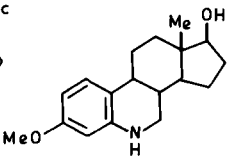
(9)



(10)



(11)



(12)

the naturally occurring heterosteroids, a mention may also be made of two antibiotics, viridin (9) [40] and wortmannin (10) [41], produced by *Gliocladium virens* and *Penicillium wortmanni*, respectively. The cardioactive cardenolides and bufadienolides are well-known. To the latter class belong the steroid constituents of toad venom of which pharmacological and toxicological features have been reviewed [42].

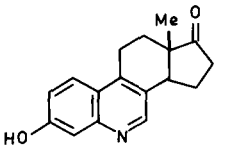
SYNTHETIC HETEROSTEROIDS

Hundreds of nucleoheterosteroids which are purely of synthetic origin have been prepared by total or partial syntheses.

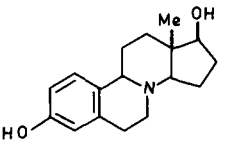
For introduction of a heteroatom(s) by total synthesis suitable mono- or bi-cyclic heterocyclic systems have been utilized to construct the heterosteroid skeleton [43]. The other approach is based on construction of heterocyclic component of the steroid nucleus through a sequence of special synthetic techniques [43]. The early synthetic references to the partial synthesis of heterosteroids were compiled by Tökés [44]. Among available other reviews may be mentioned the one by Ninomiya [45] on azasteroids and by Pappo [46] and Ramdas and Chaudhuri [47] on oxasteroids.

Certain genuine hetero isosteres of natural hormones have been synthesized and these include 2-azaestradiol 3-methyl ether 17-acetate (11) [48,49], (±)-2-azaestradiol 3-methyl ether [50], (±)-6-azaestradiol methyl ether (12)

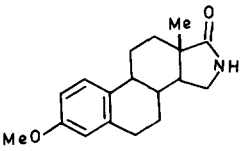
[51], (±)-6-azaequilenin (13) [52], (±)-8-azaestradiol (14) [53], (±)-8-azaestrone [54], 16-azaestrone methyl ether (15) [55,56], 17-azaprogesterone (16) [57], 2-oxatestosterone (17) [58], 2-oxaprogesterone (18) [58], 2-oxahydro-



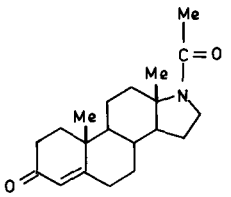
(13)



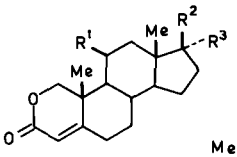
(14)

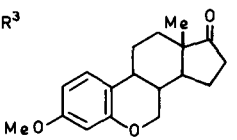


(15)



(16)

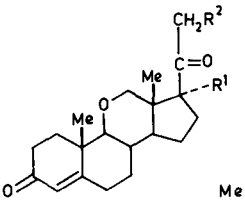


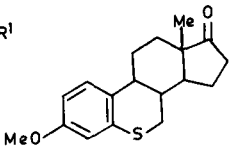


(17) $R^1 = R^3 = H$; $R^2 = OH$ (20)

(18) $R^1 = R^3 = H$; $R^2 = Ac$

(19) $R^1 = OH$; $R^2 = COCH_2OAc$; $R^3 = OH$





(21) $R^1 = R^2 = H$ (23)

(22) $R^1 = R^2 = OH$

cortisone acetate (19) [59], (±)-6-oxaestrone methyl ether (20) [60], 11-oxaprogesterone (21) [61], 11-oxaestradiol [62], 11-oxatestosterone [62], 11-oxa analogue (22) of 17*α*-hydroxydeoxycorticosterone [63], 15-oxaestrone [64], 16-oxaestrone [56], and (±)-6-thiaestrone methyl ether (23) [65]. Recently, reports on the synthesis of nucleo-selena [66,67], telluria [66,67], and phospha [68,69] heterosteroids have appeared. Regarding heterosteroids in which the heteroatom(s) forms part of the ring attached to the steroid nucleus, reference may be made to reviews by Zhungietu and Dorofeenko [70] and Akhrem and Titov [71].

HETEROSTEROIDS AND DRUG DESIGN

The literature on biological activity of heterosteroids [72], particularly the azasteroids has been the subject of some reviews [45,73—78]. From medicinal chemists' point of view, the synthetic heterosteroids may be said to have been prepared on empirical grounds but the research in certain instances in the synthesis of new heterosteroids has been the result of reasoning based on the concepts of drug design. The facets of heterosteroids to be reviewed pertain to adrenocorticoids, anti-mineralocorticoids, anabolics, androgen antagonists and biosynthesis inhibitors, oestrogenic, anti-oestrogenic, progestational, anti-progestational, anti-fertility, catatoxic, cardiac, anti-lipemic, central nervous system acting, neuromuscular blocking, local anaesthetic, diagnostic, antimicrobial, anti-neoplastic, and miscellaneous agents.

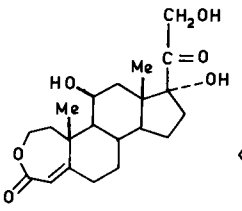
HETERO MODIFICATIONS OF ADRENOCORTICOSTEROIDS AND SOME RELATED ASPECTS

The adrenal steroids continue to be one of the most fascinating areas of endocrine research. Medicinal chemists have ventured to modify the corticosteroid structures in different ways and many highly successful drugs have resulted. The review in this section is limited to work on hetero modifications and some closely related aspects. Most of the work has been on changes in the structures of glucocorticoids, resulting in several extremely potent anti-inflammatory agents. Some heterosteroids obtained from natural sources also possess this activity. Certain hetero analogues of the mineralocorticoid deoxycorticosterone have also been prepared and examined. Some of the synthetic compounds are adrenal inhibitors and anticatabolics.

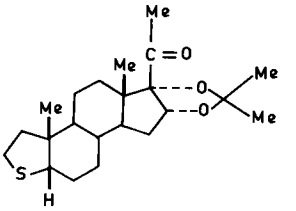
SUBSTITUTION OF NUCLEO-METHYLENE WITH HETEROATOMS

There are only a few studies with the analogues where a nuclear methylene function has been replaced by a heteroatom. This has not resulted in any compound of promise but this kind of modification is associated with anti-inflammatory activity to a certain extent.

2-Oxahydrocortisone acetate (19) is one fourth as effective as hydrocortisone in reducing the eosinophil count, in the liver glycogen deposition assay, and in the local and systemic granuloma inhibition tests [59]. The 9*α*-chloro-16*α*-methyl derivative of (19) was approximately equipotent with hydrocortisone in the liver glycogen deposition assay. 3-Oxa-A-homohydrocortisone (24) is at least as active as hydrocortisone and does not show salt retaining properties [46]. 2′,2′-Dimethyl-3-thia-A-nor-5*β*-pregnano[16*α*, 17*α*-d]-1′,3′-dioxolan-20-one (25) exhibited activity equivalent to betamethasone valerate in vasoconstrictor assay but inferior in the stripped skin assay [79].



(24)

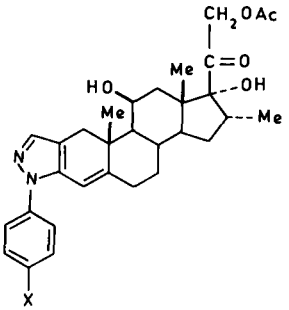


(25)

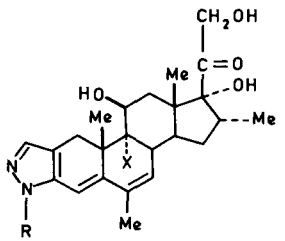
STEROIDAL [3,2-c] PYRAZOLES AND OTHER HETEROCYCLES

At a time when no potent anti-inflammatory agent lacking a C-3 oxygen function had been described, and when tampering with this position appeared to reduce the activity as seen from inactivity of 17*α*, 21-dihydroxy-4-pregneno[2,3-d]-isoxazole-11,20-dione [80], the finding about highly potent anti-inflammatory steroidal[3,2-c]pyrazoles [81,82] was unexpected. 2′-Phenyl-11*β*, 17*α*, 21-trihydroxy-16*α*-methyl-4-pregneno[3,2-c]pyrazol-20-one 21-acetate (26) and the *p*-fluorophenyl analogue (27) were found to be 60 and 100 times as active as hydrocortisone respectively in the oral granuloma assay, whereas the isomeric 1′-phenylpyrazole was less than twice as active as hydrocortisone [81,83]. 2′-(4-Fluorophenyl)-9*α*-fluoro-11*β*, 17*α*, 21-trihydroxy-16*α*-methyl-4-pregneno[3,2-c]pyrazol-20-one 21-acetate was

about 500 times as active as hydrocortisone both in inhibiting granuloma formation and in causing adrenal atrophy [81,84], whereas its 2′-unsubstituted analogue was much less active. 2′-Phenyl-9*α*-fluoro-11*β*, 17*α*, 21-trihydroxy-6, 16*α*-dimethyl-4,6-pregnadieno[3,2-c]pyrazol-20-one (28), which has a number of activity enhancing functions, has a potency 2000 times that of hydrocortisone in the rat systemic granuloma assay [82]. The 2′-*p*-fluorophenyl substituent is activity enhancing [81,83] but this potentiation cannot be related to the deactivating effect on the phenyl ring. The *p*-chloro analogue of (27) was only 15 times as active as hydrocortisone [83].



(26) X=H
(27) X=F



(28) X=F; R=

(29) X=H; R=

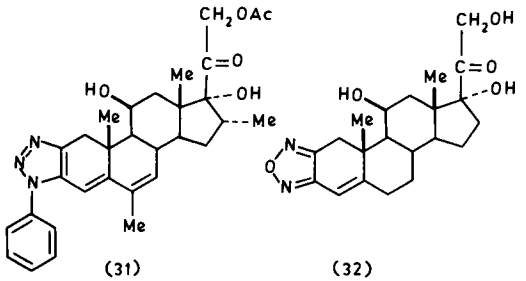
(30) X=H; R=

In the oral granuloma assay 2′-(2,4-difluorophenyl)-11*β*, 17*α*, 21-trihydroxy-6, 16*α*-dimethyl-4,6-pregnadieno[3,2-c]pyrazol-20-one proved to be about 115 times as active as hydrocortisone [85] whereas its ortho-unsubstituted analogue was 600 times as active as hydrocortisone [82]. The 21-acetate of (29) and its 21-deoxy derivative on evaluation in animals and in man for glucocorticoid activity did not exhibit a significant separation of the biological activities measured [86]. That one can drastically alter the structure

of hydrocortisone and prepare highly potent compounds which differ only quantitatively is intriguing. The corticoid activity-potentiation effect of 6,7-difluoromethylene function has been found to be operative in [3,2-c]pyrazoles as well [87]. In general, the [3,2-c]pyrazole function, unlike the 2*a*-methyl substituent, does not enhance mineralocorticoid activity [83].

2′-(4-Pyridyl)-11*β*, 17*a*, 21-trihydroxy-6, 16*a*-dimethyl-4,6-pregnadieno [3,2-c]pyrazol-20-one (30) [88] shows good separation of systemic to local activity (rat granuloma assay); it exhibits high vasoconstriction activity in human volunteers and is clinically effective in the treatment of psoriasis. The compound showed no progestational activity at 1.0 $mg \cdot kg^{-1}$ in the Clauberg — McPhail assay.

The success with [3,2-c] pyrazoles has led to the synthesis of several derivatives with other heterocycles fused to 2,3-position: steroidal[3,2-d] thiazoles, [2,3-d]imidazoles, [3,2-d]triazoles and [3,2-d]pyrimidines [89], and [2,3-c]furazan [90]. Of these, 3′-phenyl-11*β*, 17*a*, 21-trihydroxy-6, 16*a*-dimethyl-4,6-pregnadieno[3,2-d]-3′H-1′, 2′,3′-triazol-20-one 21 acetate (31) has an activity 190 times that of hydrocortisone in the rat systemic granuloma assay [89,91], and 11*β*, 17*a*, 21-trihydroxy-4-pregneno[2,3-c]furazan-20-one (32) has been found to be 2.5 times as active as hydrocortisone [90]. No activity was observed with the 2′-phenyltriazolo or the 1′-phenylpyrazolo steroid, and the structural specificity for anti-inflammatory activity is rather remarkable.

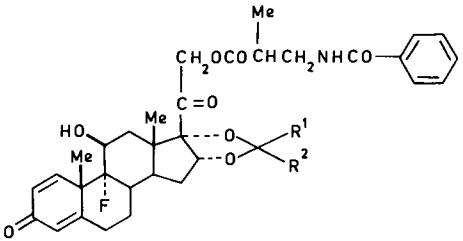


(31) (32)

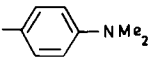
16*a*, 17*a*-FUSED HETEROCYCLES

Triamcinolone acetonide has a dioxolan system fused to positions 16*a*, 17*a*. Corresponding to this prototype, different structural changes have been made. Among the compounds obtained by esterification of C-21 hydroxyl

of triamcinolone acetonide [92], the β-benzoylaminoisobutyrate ester (TBI) (33) has been found to possess a strong anti-phlogistic activity [93]. It has given favourable results in patients affected by varied dermatoses [94] and studies in rabbits show that it is better tolerated in eye applications also [95]. The analogue (TBI/PAB) (34) has been found to be 19.8 times more potent than triamcinolone acetonide by topical application in rats [96].

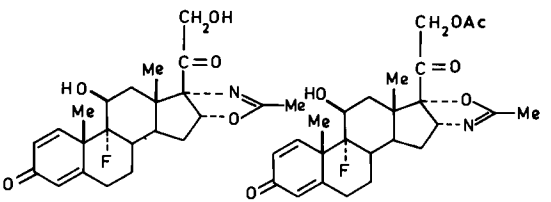


(33) $R^1 = R^2 = Me$

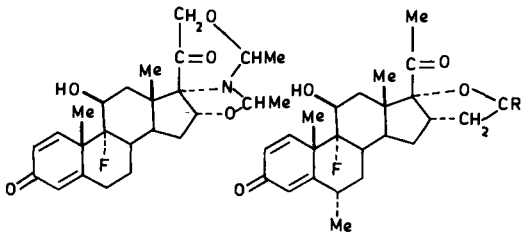
(34) $R^1 = H$; $R^2 =$ 

[17a, 16a-d]-2′-Methyloxazolino analogues of prednisolone and 9a-fluoroprednisolone are significantly active when tested for neoglycogenetic and anti-inflammatory activity in the rat [97,98]. Compound (35) was 180 and 250 times more potent than hydrocortisone in these tests. It has been shown that the 21-acetate (fluazacort acetate; L6400) of (35) like triamcinolone acetonide is poorly absorbed through the skin in rats, pigs and humans [99]. The isomer (36) of fluazacort acetate was also prepared but it was less active [100]. The related hexacyclic corticoid (37) had high topical, as well as high systemic, activity in the rat [101].

A considerable variation in the heterocycle fused to 16, 17 seems to be permissible for retention of the activity. 9a-Fluoro-11β, 17a-dihydroxy-



(35) (36)



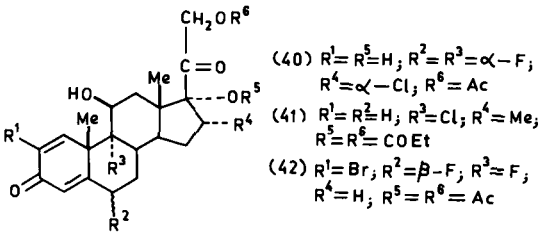
(37) (38) R = O
(39) R = H, OH

6α-methyl-3,20-dioxo-1,4-pregnadien-16α-acetic acid γ-lactone (38) showed an anti-inflammatory activity 72 times that of hydrocortisone (subcutaneous) in the rat granuloma pouch assay, while the lactol (39) was 33 times as active as hydrocortisone in this test [102]. The lactone was also 63 times as active as (subcutaneous) hydrocortisone in the rat glycogen deposition assay but inactive as a mineralocorticoid.

SUBSTITUTIONS OF THE STEROID NUCLEUS POSITIONS

Several corticoids having steroid nuclear skeleton substituted with different halogeno groups are well-known. Many of the fluoro derivatives are used in medical practice. There have been also obtained a number of derivatives possessing substituents like amino, azido, methylthio, thiocyanato, isothiocyanato groups. Some examples may be cited.

Among the 16α-chloro corticoids studied [103], 16α-chloro-6α, 9α-difluoroprednisolone 21-acetate (40) was 1100 times as potent as hydrocortisone in the granuloma pouch anti-inflammatory assay in rats and 1030 times the activity of hydrocortisone in liver glycogen deposition assay. Beclomethasone dipropionate (41) is a 9α-chloro derivative which is an anti-



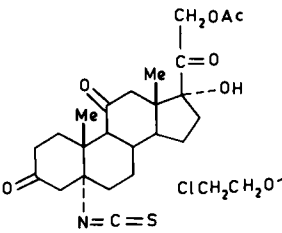
(40) $R^1 = R^5 = H$; $R^2 = R^3 = \alpha\text{-F}$; $R^4 = \alpha\text{-Cl}$; $R^6 = Ac$
(41) $R^1 = R^2 = H$; $R^3 = Cl$; $R^4 = Me$; $R^5 = R^6 = COEt$
(42) $R^1 = Br$; $R^2 = \beta\text{-F}$; $R^3 = F$; $R^4 = H$; $R^5 = R^6 = Ac$

asthmatic agent of interest [104—107]. Halopredone acetate (42) is a new topical anti-inflammatory steroid under study [108].

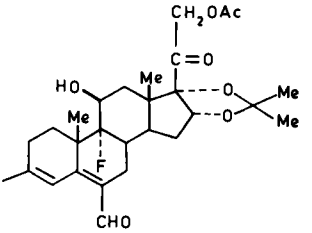
Certain 16β-amino-17α-hydroxy-20-oxopregnenes exhibited anti-inflammatory activity [109] which appeared to be increased by adding methyl groups to the 16β-nitrogen. 17β-Amino-3,5-androstadiene and 17β-amino-5-androstene were active as anti-inflammatory agents (against rat hind paw oedema), and the *N*-substituted derivatives 17β-(3-aminopropylamino)-3, 5-androstadiene and 17β-(3-aminopropylamino)-5-androstene were more potent [110]. 6-Azido-6-dehydro analogue of hydrocortisone acetate was shown to be 4—8 times as active as the latter (rat granuloma pouch) [111].

2α-Methylthiocortisone caused an increase in sodium and urine excretion but was only less than one-half as active as hydrocortisone according to liver glycogen and thymolytic assays [112]. 9α-Thiocyanatohydrocortisone acetate and 9α-thiocyanatocortisone acetate have shown no appreciable corticoid activity [113,114].

5α-Isothiocyanato analogue (43) showed the same order of glucocorticoid activity as cortisone acetate — may be it is converted to the 4-en-3-one *in vivo* [115]. The 11β-hydroxy compound corresponding to (43) was only one-fourth to one-tenth of the parent hydrocortisone acetate in activity. The compound (44) was shown to be active at low doses in protecting mice against fatal anaphylactic shock (experimental allergy) [116].



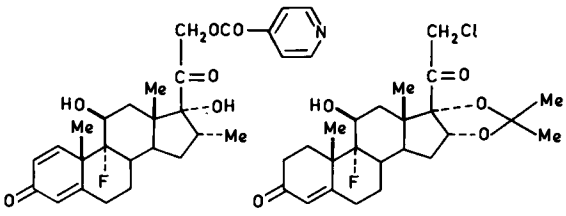
(43)



(44)

MODIFICATIONS OF THE 21-CH_2OH FUNCTION

The—CH_2OH of the 20,21-ketol function has been modified in different ways. One common change is the preparation of different esters of the primary alcoholic function; the substitution of the—OH group, and structural modifications involving or substituting C-21 carbon have also been found to be of interest. Co-ordination copper complexes of hydrocortisone



(45) (46)

21-phosphate, hydrocortisone 21-hemisuccinate and dexamethasone 21-phosphate are active in anti-ulcer effects [117,118].

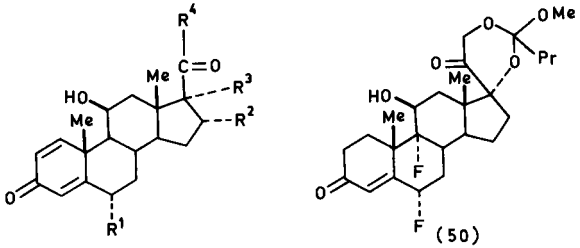
Dexamethasone pyridine-4-carboxylic acid ester (HE 111, Auxiloson) (45) has been found to be several times more potent than the parent dexamethasone in different standard tests for glucocorticoid and anti-inflammatory activities in rats [119,120]. Because of the stability of carbamate esters to hydrolysis, some 21-carbamates of corticosteroids were prepared and examined [121]. Most of these esters were active in the local granuloma inhibition assay but were less active in systemic tests. The C-21 nitrate esters are of no significant interest in terms of the corticoid activity [122].

Several 21-chloro derivatives have been studied [123—125]. Of particular interest are the clinically useful anti-inflammatory agents, 21-chloro-9α-fluoro-11β-hydroxy-16β-methyl-17α-propionyloxy-1,4-pregnadiene-3,20-dione (clobetasol propionate) and halcinonide (46) [127,128]. Clobetasol propionate has been tried in the treatment of vitiligo [129].

21-Iododeoxycortisone and cortisone 21-iodoacetate have been examined and offered for possible application to study certain steroid binding sites of high affinity present in receptor proteins of target organs [130].

There are reported cases where there is retention of biological activity of corticoids on replacement of 21-hydroxy with nitrogen functions. Thymus involution activity (approximately equal to that of hydrocortisone) has been demonstrated for 21-amino-9α-fluoro-11β, 17α-dihydroxy-4-pregnene-3,20-dione [131]. 21-Deoxy-21-amino analogues have been shown to be active, particularly 21-piperidino [132,133] and 21-piperazino derivatives [134].

Mazipredone (Depersolone), chemically 11β, 17α-dihydroxy-21-(4-methyl-piperazin-1-yl)-1,4-pregnadiene-3,20-dione (47) hydrochloride, possesses marked glucocorticoid and anti-inflammatory activity [134]. It showed better protective action than prednisolone hemisuccinate, using the intravenous route, against anaphylactic shock induced in guinea-pigs by an albumin aerosol [135]. Studies in rats showed that mazipredone was well absorbed after administration by all routes [136]. A metabolic product



(47) $R^1 = R^2 = H$; $R^3 = OH$; $R^4 = CH_2N$ N–Me

(48) $R^1 = R^2 = H$; $R^3 = OH$; $R^4 = -CH$ N, O

(49) $R^1 = F$; $R^2 = Me$; $R^3 = H$; $R^4 = -COOBu$

in humans has been tentatively identified as prednisolone [137]. However, prednisolone as a metabolite seems to play a minor role in mazipredone action, since a rather low conversion rate of mazipredone to prednisolone and significantly different half-lives of the two hormones were observed.

Esters of 21-mercapto analogues have been claimed to possess anti-inflammatory activity [138], as also have 21-aldehyde hydrates [139] and acetals [140]. A corresponding oxazolidine derivative (48) was more active than hydrocortisone in thymolytic assay [141].

Conversion of $-CH_2OH$ to $-COOR$ retains the anti-inflammatory activity. The esters of 6*α*-fluoro-11*β*-hydroxy-16*α*-methyl-3, 20-dioxo-1,4-pregnadien-21-oic acid were found to possess a local anti-inflammatory activity [142]. The butyl ester (49) (fluocortin butyl ester, Vaspit) [143] has been found to be of clinical interest [144,145].

Hydroxamic acid analogues of cortisone (—NHOH and—NHOMe in place of 21-CH_2OH) were inactive in thymus involution and pellet granuloma tests [146]. In adrenalectomized rats they showed no anti-corticoid activity when given concurrently with hydrocortisone.

A mention may be made of 17-spirodioxans which involve 17*α*- and 21-hydroxyls [147,148]. The compound (50) possesses 10 times the activity of 6*α*, 9*α*-difluorohydrocortisone [147]. It may be noted that anti-inflammatory properties have also been found for 17-spirolactone [149].

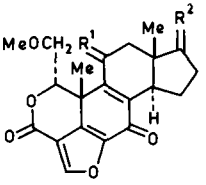
ANTI-INFLAMMATORY HETEROSTEROIDS OF NATURAL ORIGIN

The steroid glycoalkaloid, tomatine, has been shown to possess potent anti-inflammatory activity in different test systems, but its aglycone tomati-

dine has not exhibited significant inhibitory effects in any of the tests used [150]. Tomatine is a relatively nontoxic agent [151], and should it prove to be effective as an anti-inflammatory agent in humans without exerting side effects, it may be of use in the treatment of chronic inflammatory diseases.

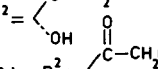
α-Solanine and to a lesser extent g-strophanthin (ouabain) have also been shown to possess anti-inflammatory effects in rats [152]. The former possibly acts directly on the adrenal cortex whereas g-strophanthin appears to act via the hypophysis.

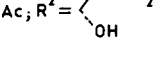
11-Deacetoxywortmannin (51), a metabolite isolated from culture filtrate of *Penicillium funiculosum Thom.*, has been shown to be a highly active anti-inflammatory agent [153]. Its activity in inhibiting the carrageenin paw oedema is about one-fourth that of dexamethasone. Analogues (52—54) gave similar results.

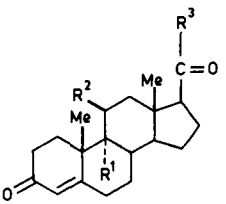


(51) $R^1 = H, H$; $R^2 = O$

(52) $R^1 = O$; $R^2 = O$ $\overset{O}{\overset{\|}{C}}-CH_2OAc$

(53) $R^1 = H, H$; $R^2 =$ 

(54) $R^1 = H, \alpha\text{-}OAc$; $R^2 =$ 



(55) $R^1 = R^2 = H$; $R^3 = CH_2NHAc$

(56) $R^1 = Br$; $R^2 = OH$; $R^3 = CHN_2$

DEOXYCORTICOSTERONE ANALOGUES

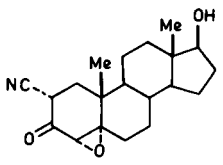
There are a few reports on the analogues of deoxycorticosterone which is a mineralocorticoid. The 21-acetylamino derivative (55) is an active mineralocorticoid in adrenalectomized rats, possessing no progestational

(Clauberg test, 1000 μg dose) or deoxycorticosterone antagonist activity (Kagawa test) [154]; but the 21-deoxy-21-piperidino analogue of deoxycorticosterone has weak mineralocorticoid activity [155]. The deoxycorticosterone analogue 8,21-dihydroxy-3,20-dioxo-4-pregnen-19-oic acid 19,8-lactone and its 17α-isomer are devoid of mineralocorticoid activity in rats [156].

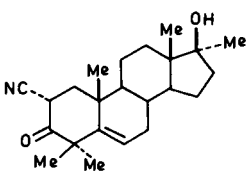
Interestingly, the nitrate ester of deoxycorticosterone has been reported to promote protein metabolism [122]. The 21-diazo derivatives like 9α-fluoro and 9α-bromo-21-deoxycorticosterone, 21-deoxycorticosterone, and progesterone are of interest for use as photoaffinity labels for mineralocorticoid receptors [157]. In the isolated toad bladder system, the compound (56) was as active as aldosterone and more active than 9α-fluorohydrocortisone in augmenting active Na^+ transport.

SOME ADRENAL INHIBITORS AND ANTI-CATABOLICS

Through increased output of corticosteroids by the adrenal cortex, ACTH treatment results in an increased excretion of nitrogen in the urine and a reduction in the weight of thymus. Some compounds like (57, Trilostane) have been described [158] which block the ACTH-induced catabolic and thymolytic responses in castrated rats. Derivatives of (57) with changes at position 17, such as the acetate, 17α-methyl, 17α-ethinyl, hemisuccinate, vary in degree of lipid and water solubility but possess marked adrenal blocking activity. The analogue (58) has been found to inhibit the enzyme(s) responsible for conversion of 3β-hydroxy-5-en-steroids to the corresponding 4-en-3-one [159—161]. As such, this compound has proved useful in stimulating a variant of congenital adrenal hyperplasia in experimental animals [162]. In addition to inhibitory effects on 3β-hydroxysteroid dehydrogenase, the cyanoketone has been shown to inhibit cholesterol side chain cleavage during incubations of adrenal homogenates or sections [163]. This effect on cholesterol side-chain cleavage may be partly due to pregnenolone accumulation with resultant product inhibition. It appears that



(57)



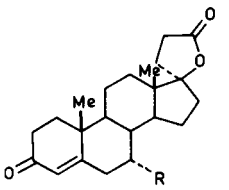
(58)

the cyanoketone must inhibit desmolase activity by a mechanism which is independent of pregnenolone feed-back inhibition. 6-Azido-17*α*-hydroxy-16-methylene-4,6-pregnadiene-3,20-dione 17-acetate showed striking adrenal suppression activity in rats [111].

Anabolic agents are useful in offsetting the catabolic effects observed clinically following chronic administration of glucocorticoids. The latter produce marked increase in tryptophan pyrrolase activity in rat liver. The anabolic steroid 17*β*-hydroxy-17*α*-methyl-5*α*-androstano[3,2-c]pyrazole (85) exhibited the highest activity in suppressing tryptophan pyrrolase induction (30 $mg \cdot kg^{-1} \times 2$); whereas methyltestosterone produced no suppression at 240 $mg \cdot kg^{-1} \times 2$ [164].

HETEROSTEROIDAL ANTI-MINERALOCORTICOIDS

In 1957, the G.D. Searle research workers reported a significant finding about the anti-mineralocorticoid activity of the spirolactones of the type (59) [165,166]. This evoked the synthesis of several modified spirolactones and certain other 17-spiro hetero systems with or without changes in the steroid skeleton. This line of thought led to the development of the useful drug spironolactone (60) (Aldactone) [167].



(59) R = H

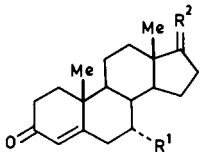
(60) R = SAc

DIFFERENT 17-SPIRO HETERO SYSTEMS

The spiro system present in (59) and (60) has been modified in different ways. Stereochemical importance of C-17 was demonstrated through observed inactivity of 21-carboxy-17*α*-hydroxy-4-pregnen-3-one lactone [168,169] as an aldosterone blocker in contrast to the activity of (59). Lactone carbonyl seems to be a non-essential function, for the derivative (61) when adminis-

tered orally to rats has about the same aldosterone antagonist activity as the corresponding lactone [170]. Lactols of the type (62) have also been prepared [171,172].

There is a fall in the activity on introduction of a double bond at C-20 to C-21 or expansion of the lactone ring to a six-membered ring [173]. Certain other variations have led to inactive compounds such as (63,64) [174,175], (65) [176,177], and (66) [178—180]. The spiro oxazolidine derivative (67) showed weak deoxycorticosterone-blocking activity [181]. Activity has been claimed for the analogue (68) [182] and (69) [183]. The analogues related to (70) (X = OH, OAc, H) and anhydride (71) were inactive as inhibitors of aldosterone in adrenalectomized rats [184]. Spiro sulphinic acid lactones have been claimed to inhibit aldosteronism in rats [185].

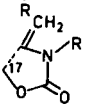


(61) $R^1 = SAc$; $R^2 =$ 

(62) $R^1 = H$; $R^2 =$ 

(63) $R^1 = H$; $R^2 =$ 

(64) $R^1 = H$; $R^2 =$ 

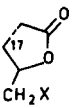
(65) $R^1 = H$; $R^2 =$ 

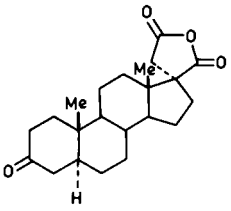
(66) $R^1 = H$; $R^2 =$ 

(67) $R^1 = H$; $R^2 =$ 

(68) $R^1 = H$; $R^2 =$ 

(69) $R^1 = H$; $R^2 =$ 

(70) $R^1 = H$; $R^2 =$ 



(71)

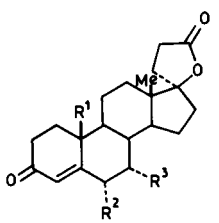
17-SPIROLACTONES WITH MODIFICATIONS IN THE STEROID NUCLEUS

The 17-spirolactone system (59) as reported originally [165] proved to be more fruitful for study of the aldosterone antagonists. Different analogues which have the same 17-spirolactone system but differ in the steroid nucleus have been prepared.

Changing the 3-oxo-4-ene system in (59) to a 3-hydroxy-5-ene, 3-oxo-4,5-dihydro, or an aromatic ring A reduces the antideoxycorticosterone and anti-aldosterone activities [173]. 3-(17β-Hydroxy-1,4-dimethyl-1,3,5(10)-estratrien-17α-yl)-propionic acid lactone was inactive [186]. On the other hand, passing from the normal series (59) to the 19-nor compound enhances the activity [165,173]. The 19-nor-steroid (72) was the most active. The 3-deoxyspirolactones, 3-(17β-hydroxy-2-androsten-17α-yl) propionic acid lactone and 3-(2-formyl-17β-hydroxy-2-androsten-17α-yl)propionic acid lactone, have been prepared, but no activity has been indicated [187]. 2,3α- and 2,3β-Epithia-5α-androstane-17-spirolactones have not shown any measurable anti-aldosterone activity [188].

In 17-spirolactone (59), introduction of unsaturation at 1,2-, 6,7-, or both, and substitution of 1α- and 7α-acetylthio grouping enhances the oral aldosterone blocking activity [167]. The 7α-acetylthio analgoue (60) (spironolactone) was found to be the most potent compound when administered orally. This was unexpected since spironolactone failed to show improved activity over (59) when administered parenterally. The aldosterone blocking activity of the 6α-methyl derivative (73) is the same as that of spironolactone (60), given subcutaneously to adrenalectomized rats [189]. The 7β-acetylthio isomer of spironolactone has 10% of the activity of latter; both oral and parenteral activities are lowered.

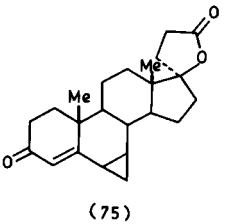
6α-Carboxymethyl derivative of (59) is claimed to be active [190]. Among the 7α-alkoxycarbonyl spirolactones (74), the methyl, ethyl and isopropyl analogues as well as the C-1 unsaturated methyl ester showed good oral and subcutaneous activity as deoxycorticosterone acetate antagonists in



(72) $R^1 = R^2 = R^3 = H$

(73) $R^1 = R^2 = Me$; $R^3 = SAc$

(74) $R^1 = Me$; $R^2 = H$; $R^3 = -C(=O)-OR$



(75)

the adrenalectomized rat [191]. The substituent at position 7 must be on the *a* face as the 7*β*-methoxycarbonyl compound and its C-6 unsaturated derivative have low potency. Substitution at position 6 of (74) is not of any advantage [192]. The derivatives corresponding to (74) but lacking a lactone carbonyl have also been reported [193]. 7*α*-Cyano and 7*α*-(methoxycarbonyl) amino derivatives of (59) are claimed to be active [194].

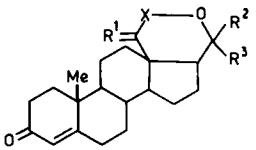
A derivative with a *β*-methylene group at the 6,7-position (75) has an activity somewhat greater than that of spironolactone [195]. An inactive metabolite of spironolactone contains a hydroxyl group at the 6*β*-position [196]. It is speculated that blockage of the hydroxylation at the 6*β*-position may be the process by which the 6*β*,7*β*-methylene group exerts its beneficial effect in the spirolactone (75) [197]. The open spirolactone analogue prorenoate, potassium 3-(17*β*-hydroxy-6*β*,7*β*-methylen-3-oxo-4-androsten-17*α*-yl)propionate, possesses the pharmacological characteristics of aldosterone antagonist similar to, but more potent than, that of spironolactone [198].

In steroidal 17-spirolactones, oxygenation of position 11 produces some increase of aldosterone-blocking activity and this activity is further enhanced by the additional introduction of 9*α*-fluoro substitution [199]. The most active compound in the series studied was 3-(9*α*-fluoro-17*β*-hydroxy-3,11-dioxo-4-androsten-17*α*-yl) propionic acid lactone. This prompted the synthesis of other 17-spirolactones bearing substituents at positions 9 and 11, but none proved to be better [200].

Several spirolactones having a hydroxyl group at various positions in the molecule have been prepared but no encouraging results with regard to anti-mineralocorticoid effects have been obtained [199,201,202].

SOME OTHER DERIVATIVES

Steroid derivatives having no 17-spiro system have also been studied for anti-mineralocorticoid activity. The tetrahydro-oxazino analogue (76) has



(76) $R^1 = O$; $X = NH$; $R^2 = \alpha$-Me; $R^3 = H$

(77) $R^1 = H, H$; $X = CH_2$; $R^2 = Me$; $R^3 = OH$

one-half of the anti-mineralocorticoid activity of spironolactone when administered parenterally [203]. 18-Hydroxymethylprogesterone, which exists in its hemiketal form (77), showed one-fourth of the activity of spironolactone [204].

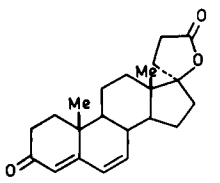
16α-Acetylthio-3β-hydroxy-5α-pregnan-20-one, the 16α-thioester analogue of the Neher's sodium excreting factor (3β, 16α-dihydroxy-5α-pregnan-20-one) was devoid of mineralocorticoid activity; the 5β, 16α-thioester analogue, 16α-acetylthio-3β-hydroxy-5β-pregnan-20-one, and its 5β, 16β-thioester epimer, 16β-acetylthio-3β-hydroxy-5β-pregnan-20-one, antagonised the action of deoxycorticosterone acetate in adrenalectomized rats [205]. 21-Hydroxy-3,20-dioxo-4-pregnene-19-nitrile 21-acetate showed no deoxycorticosterone acetate-antagonist property [206].

Compound (57) which inhibits the conversion of pregnenolone to progesterone (and hence aldosterone) has been successfully used to control primary hyperaldosteronism [207].

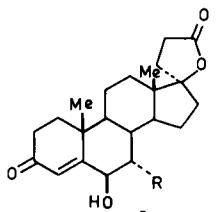
SPIRONOLACTONE

The usefulness of spironolactone (60) for diuretic therapy in oedematous patients with associated hyperaldosteronism is well established [208—210]. It has been recommended as a successful mountain sickness prophylactic [211,212].

The major metabolite of spironolactone in humans [196,213—217] and in animals [218—222] is canrenone (78). Canrenone had been synthesized earlier [167]. Besides canrenone, the other significant metabolites isolated are the 6β-hydroxy-7α-methylsulphoxide (79) [196] and 6β-hydroxy-7α-methylsulphone (80) [223,224]. 15α-Hydroxycanrenone has been isolated as



(78)



(79) R = ---S(=O)Me

(80) R = ---S(=O)(=O)Me

a spironolactone metabolite in man [225] and has also been prepared by microbiological oxidation of canrenone [226]. It does not show antimineralocorticoid activity; however, 15-oxocanrenone exhibited weak activity [226].

Potassium canrenoate (SC-14266, Soldactone), the potassium salt of the hydroxy acid derived by opening the γ-lactone ring in canrenone (78) is a water-soluble anti-aldosterone agent used as diuretic [227]. Canrenone is considered to be the actual active agent of the aldosterone antagonists spironolactone and potassium canrenoate [228,229], but it has been suggested that the activity may be significantly attributable to metabolites other than canrenone also [230—232].

In decompensated liver disease, the metabolism of spironolactone is unaltered [233]. Diuretic overtreatment in the course of mobilisation of ascites, however, might cause considerable impairment of elimination. An interruption of enterohepatic circulation has been shown to result in a shift of metabolic pathways of spironolactone, in spite of an unchanged overall metabolic rate [234].

Spironolactone and canrenone are not only competitive antagonists of aldosterone in the kidney but also inhibitors of aldosterone production in the adrenal in man [228,235,236]. Canrenone caused a significant decrease in aldosterone excretion in thiabutazide-induced hyperaldosteronism [235]. The mechanism of inhibition by canrenone in rat adrenals *in vitro* was shown to be a competitive antagonism between canrenone and precursors of aldosterone synthesis for mitochondrial hydroxylases [237]. Canrenone proved to be the more potent inhibitor of aldosterone synthesis in an identical molar concentration as compared with that of spironolactone, which also inhibits aldosterone synthesis in rat adrenals [238].

The inhibition of aldosterone synthesis is not selective since the synthesis of corticosterone is also inhibited dose-dependently by spirolactones. The spirolactone acts as a false precursor and itself is hydroxylated in the 11- or 18-position. Studies with SC-5233 (59) and 11β-hydroxylated SC-5233 on the synthesis of corticosteroids in the rat adrenal tissue [239] showed that 11β-hydroxylated SC-5233 induced a largely selective inhibition of aldosterone production; in contrast, SC-5233 had an unselective inhibitory effect on the synthesis of both corticosteroids, aldosterone and corticosterone. Spironolactone has been shown to diminish the activity of adrenal mitochondrial as well as microsomal cytochrome P-450-containing enzymes, resulting in a fall in corticosteroid output [240].

Spironolactone is receiving increased attention as a hypotensive drug in the treatment of essential hypertension [241—248], even though excessive

aldosterone is not directly involved. The drug has been used to show that renin-angiotensin-aldosterone system may be involved in primary hypertension [249,250]. In man, a positive inotropic action [251] and immunosuppressive activity [252—254] have been reported for spironolactone. Later, the anti-androgenic and catatoxic actions of spironolactone are discussed.

ANABOLIC HETERO DERIVATIVES

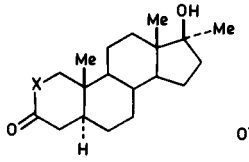
The medicinal chemists have modified the structure of testosterone in several ways with the object of dissociating the anabolic potency from undesirable masculinizing effects. Assuming that the target receptors at the sites of anabolic and androgenic activities differed sufficiently, one could conceive of preparing substances with greater specificity.

One successful approach has been of introducing hetero atoms in place of nuclear methylene functions. Further, the relative distance between substituents at the 3-position and the 17-position of the steroid nucleus, as measured by receptor site bonding to these positions ('terminal bonding'), could be altered, and/or one can aim at change in the type and stability of receptor site bonding at either position. The latter effect could be observed by substituting a more highly nucleophilic atom or group for oxygen at 3 or 17. However, this type of substitution must not change cellular absorption patterns or fail to meet the steric requirements of the receptor system. This kind of thinking motivated various synthetic schemes and different ring A fused heterocycles have been obtained. The above and other assorted type of hetero modifications are reviewed.

NUCLEOHETEROSTEROIDS

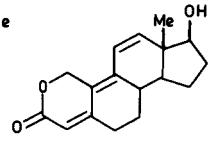
Several nucleoheterosteroids have been tested for androgenic-anabolic activity. The incorporation of heteroatom(s) has been mostly in ring A, keeping the six-membered size or its enlargement to seven-membered and reduction to five-membered systems. There have been obtained some active entities.

A compound of particular interest is oxandrolone (Anavar), chemically 17β-hydroxy-17α-methyl-2-oxa-5α-androstan-3-one (81) [58]. It is an effective anabolic agent and has been the subject of several clinical studies [255—261]. This steroid is being used frequently in the U.S.A. for muscle development in athletes [262] and as a growth stimulant for stunted children [260]. Oxandrolone is relatively resistant to metabolic transformation and has a high degree of protein binding [263].



(81) X = O

(83) X = NH

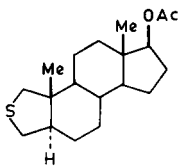


(82)

The trienic lactone (82) on oral evaluation in rats was shown to be 20 times as anabolic as methyltestosterone [46]. 17β-Hydroxy-17α-methyl-2-oxa-4,9(10)-estradien-3-one is also claimed to possess very high anabolic activity [264]. Though in (81) and (82) lactone function is compatible with anabolic activity, the analogues 17β-hydroxy-17α-methyl-3-oxa-A homo-4a-androsten-4-one [46] and 17β-benzoyloxy-4-oxa-5α-androstan-3-one [265] have not been of interest.

17β-Hydroxy-2-oxa-4,9 (10)-estradien-3-one and 17β-hydroxyl-17α-methyl-2-oxa-4,9 (10)-estradien-3-one have been shown to inhibit binding of 5α-dihydro-testosterone to receptor proteins from rat prostate tissue [266]. The 2-aza isostere (83) of oxandrolone appears to be equally active clinically [46] but 4-oxo-3-aza-A-homo-4a-en [267,268] and 3-oxo-4-aza-5-en-lactams [269] have shown weak or no activity.

Wolff and coworkers have carried interesting studies on androgenic-anabolic activities of nucleoheterosteroids. It was observed that the thia-steroid (84) had the potency of the order of testosterone [270]. Possibly the steric effects and not electronic factors are important in connection with C-2 and/or C-3 in androgens. Biological activity of 7α-methyl, 19-nor, and 17α-alkyl derivatives of 2-thia-A-nor-5α-androstan-17β-ol parallels their effects in the testosterone series [271], and the sulphoxide and sulphone related to (84), which are its most likely metabolites, are devoid of activity. It may be noted that 3-oxa-A-nor-5α-androstan-17β-yl acetate [272], 2,3-diaza-A-nor-1,3-androstadien-17β-ol [273], and 4-oxa-5α-androstan-17β-yl acetate [274] are reported to be inactive as androgenic-myogenic agents.



(84)

In contrast to activity of (84), 2-oxa-A-nor-5α-androstan-17β-ol is inactive [275]. This reflects the fact that S is isosteric with —CH = CH—. For the activity, minimum ring size is a six-membered ring or its isosteric equivalent, as selena and telluria A-nor steroids are also active [276]. The concept that the activity-engendering function is primarily steric also draws support from the observed androgenic-myotrophic activity of 17β-hydroxy-2,4-dioxa-3-thia-5α-androstan-3α-oxide acetate (50—100% of testosterone) [277].

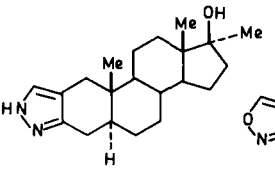
2-Oxa-5α-androstan-17β-ol (activity 50—100% of testosterone) [275] and 2-thia-5α-androstan-17β-ol [67] are both active. The latter showed one-fifth the androgenic activity of testosterone, but the levator ani response was nearly as high as that of testosterone. 3-Oxa-5α-androstan-17β-ol is less active than the 2-oxa compound and the 4-oxa derivative is inactive [275].

Out of 3-oxa-, 3-thia-, 3-selena-, 3-telluria-A-homo-5α-androstan-17β-ol derivatives and the 3,4-dithia-A-dihomo analogue (but for the telluria compound), all show both androgenic and myotrophic activity [66]. The thia derivative, 3-thia-A-homo-5α-androstan-17β-ol, is the most active, showing levator ani activity equivalent to testosterone but weaker seminal vesicle effects. An appropriate conformation of expanded ring A may be significant for the activity.

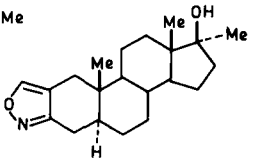
It has been reported that the incorporation of a 7-oxa function into 17β-hydroxy-17α-methyl-5α-androstane derivatives greatly diminishes their primary endocrinological properties [278]. 11-Oxatestosterone has low androgenic-anabolic activity [62]. 8-Aza-19-normethyltestosterone has one-hundredth the androgenic property of testosterone propionate and even less anabolic [279]. A mention may be made of B-secosteroid 5,6-seco-6,6-dimethyl-6-fluoro-6-sila-17β-hydroxy-estra-1 (10)-en-3-one which showed weak androgenic activity in rat [280].

RING A FUSED HETEROCYCLES

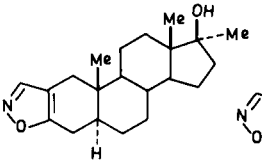
Different A ring fused heterocycles have been obtained with a view to obtain potent anabolic agents. Sterling—Winthrop workers [281—283] prepared several steroid[3,2-c]pyrazoles, and one of the compounds 17β-hydroxy-17α-methyl-5α-androstano[3,2-c]pyrazole (stanozolol, androstanazole; Winstrol) (85) has proved to be a successful anabolic agent for clinical use [284]. 17β-Hydroxy-5α-androstano[3,2-c]pyrazole and (85) were reported to have anabolic/androgenic ratio of 8 and 120, respectively, in rats [282]. Introduction of 6α-methyl or 9α-fluoro-11β-hydroxy



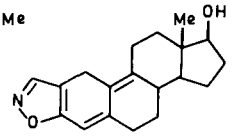
(85)



(86)



(87)



(88)

groups considerably lowered the androgenic and anabolic activity. This was unexpected since similar substituents into 17α-methyltestosterone markedly increased the activities. Putting of 7α-alkylthio substituent in stanozolol, as for example, 7α-ethylthio-17β-hydroxy-17α-methyl-5α-androstano [3,2-c] pyrazole, resulted in a better separation of myotrophic and androgenic activity [285]. The 7-oxa analogue, 17β-hydroxy-17α-methyl-7-oxa-5α-androstano [3,2-c] pyrazole, at higher dosage levels retains the significant antigonadotrophic properties of its 'normal' steroid analogue stanozolol while lacking the latter compound's anabolic and androgenic effects [278].

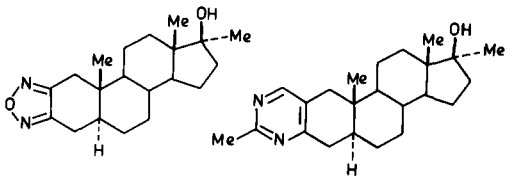
Next, analogues having isoxazole system fused to 2,3-positions were investigated. Syntex workers [80] reported 17β-hydroxy-17α-methyl-5α-androstano[3,2-c]isoxazole (androisoxazole) (86) and 17β-hydroxy-17α-methyl-4-androsteno[2,3-d]isoxazole to be highly active as anabolics and low at androgenicity; 16-hydroxy derivatives of androisoxazole have been prepared as potential metabolites [286].

The Sterling—Winthrop group [287,288] concentrated their studies on androstano[2,3-d]isoxazoles, and found 17β-hydroxy-17α-methyl-5α-androstano[2,3-d]isoxazole (87) to be the most interesting in terms of separation of anabolic from androgenic activity. It was 9.7 times as anabolic, 2 times as myotrophic and 0.24 times as androgenic as methyltestosterone when compared orally. A good number of congeners were examined, but only a limited variation could be made without greatly diminishing the degree of activity demonstrated for the 17β-hydroxy or 17β-hydroxy-17α-methyl

members. In the 19-nor series, 17*α*-methyl-19-nor-4-androsteno[2,3-d]isoxazole had progestational activity equal to that of progesterone intramuscularly and at least as active as ethisterone when given orally. 17*β*-(3-Cyclohexylpropionoxy)-5*α*-androstano[2,3-d]isoxazole was a potent anabolic agent without a long duration of action and minimal androgenicity. No oestrogenicity was observed for 17*β*-hydroxy-17*α*-methyl-4-androsteno [2,3-d]isoxazole or 17*β*-hydroxy-17*α*-methyl-4,6-androstadieno[2,3-d]isoxazole which is in contrast to the corresponding[3,2-c]pyrazoles [282]. Caspi and Piatak [289,290] also studied steroidal[2,3-d]isoxazoles. Some of the other active [2,3-d]isoxazoles include 17*β*-propoxy-3′H-cycloprop[16*α*,17*α*]-5*α*-androstano[2,3-d]isoxazole [291] and the estratrienoisoxazole (88) [292].

Several other azoles prepared include androstane derivatives having fused [3,2-d]oxazole, [2,3-d]oxazole and [2,3-d]imidazole [293], [3,2-d]thiazole [80, 294—296], and [2,3-d]triazole [293,297,298]. 17*β*-Hydroxy-17*α*,2′-dimethyl-5*α*-androstano[3,2-d]thiazole has 2 times the anabolic and 0.4 times the androgenic activity on oral administration in rats as compared with methyltestosterone [294]. The corresponding [3,2-d]oxazole showed weak myotrophic and androgenic activities [293].

Furazabol, originally introduced as androfurazanol, is another useful anabolic agent. Furazabol, chemically 17*β*-hydroxy-17*α*-methyl-5*α*-androstano[2,3-c]-1′,2′,5′-oxadiazole (89) was first prepared by Japanese workers [299—301] and latter also obtained by Havranek, Hoey and Baeder [302]. Endocrinological studies [300,303,304] showed furazabol to be a potent acceptable anabolic steroid with relatively low androgenicity. By virtue of its favourable biological activities, furazabol is now used clinically as an anabolic agent. Metabolism of furazabol in rats has been studied [305] and synthesis of labelled compounds related to the metabolites has been made [306]. Derivatives of parenterally active 17*β*-hydroxy-5*α*-androstano[2,3-c]-1′,2′,5′-oxadiazole with different substitutions at 17*α*- and 16-positions have been studied but no better compound than the parent substance has been obtained [307,308]. Conversion of 17*β*-hydroxy-5*α*-androstano[2,3-c]-1′,2′,5′-oxadiazole into 17-oxo derivative by rat liver *in*



(89) (90)

vitro has been shown [309]. This is analogous to a similar change for testosterone, apparently the furazan ring has no significant effect on the metabolic oxidation at this position.

Here, a mention may be made of the studies of the action of the anabolic steroids, stanozolol (85) and furazabol (89), on coagulo-fibrinolytic systems. Patients receiving stanozolol and dicoumarol anticoagulant therapy showed prolonged thrombotest times [310]. Administration of stanozolol and phenformin to survivors of vascular accidents exhibited an increase in fibrinolytic activity (although it was not spectacular) as well as a reduction of plasma fibrinogen [311]. A long term administration of furazabol produced a distinct increase in fibrinolytic and thrombolytic potential in the rat [312].

A series of steroidal[3,2-d]pyrimidines have been prepared [80,294, 313-316]. 17β-Hydroxy-17α-methyl-5α-androstano[3,2-d]-2′-methylpyrimidine (90) on oral administration in rats was as myotrophic and 0.33 as androgenic as methyltestosterone [315], but the desmethyl analogue, 17β-hydroxy-17α-methyl-5α-androstano[3,2-d]pyrimidine is reported to be inactive [314]. 17β-Hydroxy-5α-androstano[3,2-d]-2′,6′-diaminopyrimidine and its 17α-methyl derivative had a low order of androgenic potency and no anabolic activity [317].

Among the related derivatives may be mentioned 17β-hydroxy-5α-androstano[3,2-c]-1′,4′-thiaz-5′-en-3′-one (low androgenic-anabolic activity) [318], 17β-hydroxy-17α-methyl-5α-androstano[2,3-b]-1′,2′,5′,6′-tetrahydropyrazine (no biological data) and 17β-hydroxy-17α-methyl-5α-androstano[2,3-b]-4′H-1,4-benzothiazine (no biological data) [298].

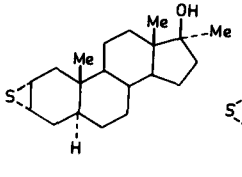
17β-Hydroxy-17α-methyl-5α-androstanol[3,2-b]pyridine [319] showed weak activity, and 17β-hydroxy-17α-methyl-5α-androstano[3,2-b]indole was inactive [320]. 17β-Hydroxy-17α-methyl-5α-androstano[3,2-b]pyrrole has also been prepared [321].

OXIRANES, THIIRANES AND DIAZIRINES

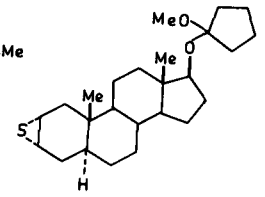
There have been prepared several steroidal ring A epoxides [322,323] and episulphides [323,324]. While the 2,3-epoxyandrostanes are not very active, pronounced activity has been found for 2,3-epithio analogues [323]. The compound (91) was found to have approximately equal androgenic and 11 times the anabolic activity of methyltestosterone after oral adminstration in castrated male rats. The 2,3-episulphide isomer of (91) was much less active.

Shionogi workers have concentrated their efforts on 2α,3α-epithioandro-

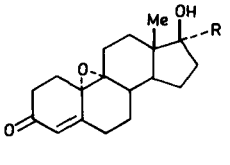
stanes. Epitiostanol (2*α*,3*α*-epithio-5*α*-androstan-17*β*-ol, 10275-S) was found to possess modest androgenic-myotrophic activities in rodents when administered subcutaneously [325,326]. On mixed acetal substitution, there was obtained 2*α*,3*α*-epithio-5*α*-androstan-17*β*-yl 1-methoxycyclopentyl ether (Mepitiostane, 10364-S) (92) [327], which was demonstrated to be more potent than the corresponding 17*α*-methyl steroid in a conventional oral assay for androgenic and myotrophic activities using rats [328]. When given orally, 10364-S is an active anabolic agent with a therapeutic potency almost equivalent to fluoxymesterone [329,330]. It is less likely to cause hepatic disturbances [331], and it has hardly any progestational activity [332]. 10364-S showed neither direct action on adrenal function nor mineralocorticoid-like activity [333].



(91)

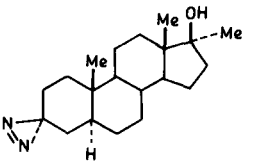


(92)



(93) R = H

(94) R = Me



(95)

17*β*-Hydroxy-9*α*,10*α*-oxido-4-estren-3-one (93) had anabolic activity approximately equal to testosterone with diminishing androgenicity when studied by subcutaneous administration in the levator ani assay, but surprisingly the 17*α*-methyl derivative (94) showed no oral anabolic activity up to 3 mg total dose [334].

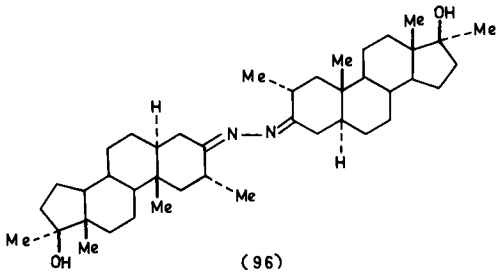
There has been reported biological data on certain spiro-oxiranes: spiro-2*β*-oxiranyl-5*α*-androstan-17*β*-ol (inactive) [322]; spiro-3*β*-oxiranyl-5*α*-androstan-17*β*-ol (inactive) and spiro-3*α*-oxiranyl-5*α*-androstan-17*β*-ol (in-

active) [335]. However, the diazirine 3,3-azo-17α-methyl-5α-androstan-17β-ol (95) showed a high anabolic-androgenic ratio on oral levator ani assay [336].

MISCELLANEOUS HETERO ANALOGUES

An indication that stanozolol (85) metabolizes to 2α-methyl-3-oxo analogue through 2α-methyl-3-hydrazone formed by cleavage of the pyrazole ring prompted the preparation of androstane 3,3′-azines [337,338], the most potent of which is 17β-hydroxy-2α,17α-dimethyl-5α-androstane 3,3′-azine (dimethazine) (96). Animal data showed dimethazine to be 10—20 times as potent as methyltestosterone on oral administration while possessing very weak androgenic action [339].

Some other workers have also prepared hydrazones [298,340,341]. The androgenic activity of testosterone 17-heptanoate could be substantially prolonged by conversion to its 3-benziloylhydrazone [341].



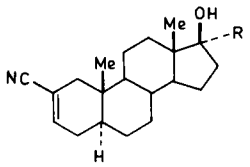
(96)

Separation of anabolic and androgenic activities has been reported in 2-(aminomethylene)-17β-hydroxy-17α-methyl-5α-androstan-3-one and certain of its nitrogen substituted derivatives [80,294]. The cysteine derivative ethyl *S*-(17β-hydroxy-17α-methyl-3,5-androstadien-3-yl)-L-cysteinate hydrochloride [342] and certain peptides prepared from 17β-amino-5α-androstan-3β-ol [343] have been reported to be inactive.

N-(3β-Hydroxy-5-androsten-17-ylidene)pyrrolidinium *p*-toluenesulphonate was weakly androgenic and myotrophic but the 3β-hydroxy-17β-pyrrolidinyl-5-androstenes [344] and 17β-dimethylamino-17-methyl-4-androsten-3-one [345] were inactive. 3β-Hydroxy-17β-pyrrolidinyl-5-androstenes were generally toxic at moderate doses, and female rats appeared to be adversely affected by lower doses than were male rats [344]. Earlier, a sex difference in the response of rats to 17-amino-4-androsten-3-one hydro-

chloride had been observed [346]. While discussing 17-substituted heterosteroids, a mention may be made of striking oral potency encountered for 17β-(tetrahydropyran-2-yl) ethers of androgen-anabolic agents [347]. The 17β-(6-substituted-tetrahydropyran-2-yl) ethers of 17β-hydroxy-2α-methyl-5α-androstan-3-one were somewhat less active than the unsubstituted THP ether [348]. Of the mono-, di-, and tritestosteroxysilanes prepared, but for trimethylsilyltestosterone no other derivative exhibited any significant androgenic or myotrophic activities [349].

Investigation of 2-cyano-2-androstenes [350—353] indicate that such modification leads to potentiation of biological activities. 2-Cyano-5α-androst-2-en-17β-ol (97) and its caproate, and 2-cyano-17α-methyl-5α-androst-2-en-17β-ol (98) are somewhat more androgenic than testosterone and methyltestosterone, respectively, but the myotrophic activity is several folds more.



(97) R=H

(98) R=Me

The observed inactivity of testosterone analogues where the 19-methyl group is replaced by a bulky function such as a cyano group [354] and derivatives such as 2β-hydroxy-19-oic lactones and 2β,19-epoxy ethers [355] having bridge across the β-face of A-ring is considered as indirect evidence of the requirement for receptor interaction of the region from the top for hormonal action. Nitro group substitution in the region of a carbonyl function in steroid hormones is derogatory for the biological activity [356]. 3β-Nitro-5α-androstan-17β-ol showed about 10% of the androgenic activity of testosterone.

Interestingly, deoxycorticosterone nitrate ester has been reported to promote protein anabolism [122], but testosterone nitrate ester was inactive.

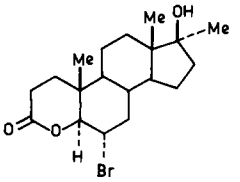
Substitution at C-4 of testosterone and 17α-methyltestosterone with halogens afforded compounds of interest. 4-Chlorotestosterone acetate (clostebol acetate, Steranabol) [357] was found to possess a strong myotrophic, low androgenic properties and, as a consequence, a high therapeutic index [358,359].

ANDROGEN ANTAGONISTS AND SOME BIOSYNTHESIS INHIBITORS

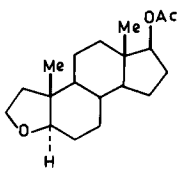
Clinically-effective androgen antagonists and biosynthesis inhibitors may presumably find use in the treatment of androgen-dependent prostatic tumours, acne, hirsutism in women, male pattern baldness, and in the treatment of hypersexuality in males. There have been reported anti-androgenic heterosteroids and some of the analogues have been shown to be inhibitors of androgen biosynthesis.

ANTI-ANDROGENS

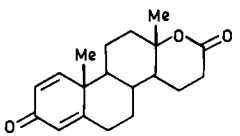
6α-Bromo-17β-hydroxy-17α-methyl-4-oxa-5α-androstan-3-one (BOMT) (99) has significant anti-androgenic activity [360,361]. The 5α- and 6α-configurations are essential and introduction of unsaturation at C-1 in (99) lowers the activity. 17,17-Dimethyl-18-nor-13-enes are reported to possess anti-androgenic activity [362], but the related analogue having 6α-bromo-3-oxo-4-oxa-5α-structure corresponding to (99) is inactive. The anti-androgenic activity of (99) is explained by its competition for the 5α-dihydrotestosterone-binding sites in androgen-dependent tissues [363]. 3-Oxa-A-nor-5α-androstan-17β-yl acetate (100), though inactive or extremely weak androgen, is effective as an anti-androgen [272,364]. It is as active as A-norprogesterone in inhibiting the hypertrophy of sex accessory organs induced by testosterone propionate [272]. Testolactone (101) antagonizes the accessory sex organ growth induced by testosterone propionate and fluoxymesterone [365].



(99)



(100)



(101)

17β-Hydroxy-4-methyl-2,3-diaza-5α-androst-3-en-1-one and 20β-hydroxy-4-methyl-2,3-diaza-5α-pregn-3-en-1-one showed mouse anti-androgenic activity 70% as compared with that of progesterone [366]. 4-Oxa-5-pregnene-3,20-dione and 4-methyl-4-aza-5-pregnene-3,20-dione possess mild anti-androgenic activity [367]. A mention may be made of the observed anti-androgenic activity of 7,8,9,10-tetrahydrobenzo[c]phenanthridine and its 7-hydroxy derivative [368] and the 7-oxo derivative [369].

Certain derivatives having heterocyclic rings fused to the steroid nucleus have been tested for anti-androgenic activity: 3,17β-dihydroxy-1,3,5(10)-estratrien-16β-yl-acetic acid lactone and 17β-hydroxy-3-oxo-4-estren-16β-yl-acetic acid lactone (appear to be anti-androgenic) [370]; (20R)-18a,20-oxido-18-homo-4-pregnen-3-one, 3-oxo-4-androsteno[17β,16β-b-]tetrahydrofuran, and 3-oxo-5α-androstano[17β,16β-b]tetrahydropyran (active) [371]; 6α-fluoro-3′-methyl-3-oxo-4-androsteno[17,16-d]pyrazole (active) [372]; 17β-acetoxy-5α-androstano[4,3-g]-2′,4′-diaminopteridine [373] and 17β-acetoxy-5α-androstano[2,3-g]-2′,4′-diaminopteridine [374] (some anti-androgenic activity); 17β-hydroxy-17α-methyl-5α-androst-2-eno[3,2-b]indole (inactive) [320].

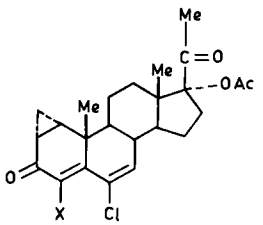
There are anti-androgenic side effects of long-term or high dose spironolactone (60) therapy. It may produce gynaecomastia in man and menstrual disturbances in women. All men in a clinical study receiving 400 mg spironolactone per day experienced painful gynaecomastia, decrease in libido, and about a 30% of the subjects complained of relative impotence [375]; semen abnormalities have also been observed [376]. Anti-androgenic effects in a controlled clinical trial of spironolactone (50 mg twice a day) in patients with benign prostatic hypertrophy have been demonstrated [377]. In castrated mice, spironolactone caused not only weight decrease in the androgen-dependent seminal vesicles but also in the kidney [378]. Direct anti-androgenic effects of spironolactone at the testosterone target organs in the castrate male rat injected with testosterone enanthate have been reported [379,380]. May be, in addition, the anti-androgenic effects of spironolactone are enhanced by suppression of plasma testosterone concentration and this suppression has been shown in man using spironolactone [381,382], the metabolite canrenone (78) [383], and potassium canrenoate [384,385]. Spironolactone has been shown to be capable of stimulating gonadotrophin secretion by interrupting negative feed-back inhibition in boys with delayed puberty [386]. The excretion of oestrogens in urine was significantly increased on administration of spironolactone [381].

Spironolactone possibly interferes with both biosynthesis and peripheral actions of androgens. It inhibits specific 5α-dihydrotestosterone-binding with

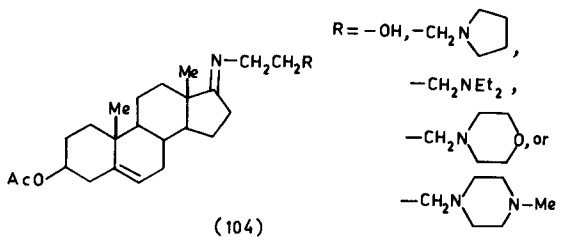
rat ventral prostate [387] and interferes with testosterone biosynthesis in rat testes by reducing 17-hydroxylase activity [388].

Spiroxasone (4′,5′-dihydro-7α-mercaptospiro[androst-4-ene-17,2′-(3′H)-furan]-3-one acetate or 7α-acetylthio-4-androstene-17(S)-spiro-2′ tetrahydrofuran-3-one) is also an anti-androgen [379]. The anti-androgenic activity associated with spironolactone and spiroxasone is increased by addition of 1,2α-methylene function and by incorporation of either a 6-chloro-6-ene or 6,7α-difluoromethylene group to the basic steroid skeleton [389], as tested in immature male castrated rats treated with testosterone enanthate.

A mention may be made of anti-androgenic halogenopregnanes. Cyproterone acetate (102) is a well-known anti-androgen [390]. A number of its derivatives [391] are active; the 4-chloro analogue (103) has pronounced anti-androgenic properties [392]. A recent study identifies 15β-hydroxycyproterone acetate as the major unconjugate metabolite of cyproterone acetate [393]. 6-Chloro-16-methylene-17α-hydroxy-4,6-pregnadiene-3,20-dione 17-acetate is an active anti-androgen [394] and the 6-azido analogue [111] also retains sufficient anti-androgenicity. Chlormadinone acetate, 17α-acetoxy-6-chloro-4,6-pregnadiene-3,20-dione (CMA), a highly active progestational agent, has potent anti-androgenic action [395]. 3β-Hydroxy-CMA, one of the main metabolites in humans and rats, and its acetate are 0.7 times as active as CMA as anti-androgenic in the rat [396].



(102) X=H
(103) X=Cl



(104)

Certain 4-alkylamino derivatives of testosterone are claimed to be anti-androgenic [397]. Some 3β-selenosteroids are also anti-androgenic [398]. Some 17-iminosteroids (104) in castrated male rats inhibited the response to testosterone propionate in the seminal vesicles and ventral prostate glands [399].

17,20-LYASE INHIBITORS

A series of 17β-acylamino-4-androsten-3-ones have been examined [400] as inhibitors of the 17,20-lyase step in the conversion of C-21 to C-17 steroids, employing an *in vitro* screening procedure which involved measurement of [^{14}C]acetate formed in the side-chain cleavage of [21-^{14}C]-17α-hydroxyprogesterone by a rat testicular microsomal preparation. Testosterone carbamate, 17β-formamido-4-androsten-3-one, 17β-acetamido-4-androsten-3-one, 17β-ureido-4-androsten-3-one, for example, were highly active and more specific than the known nonsteroidal inhibitors. None of the acylaminoandrostenones is anti-androgenic.

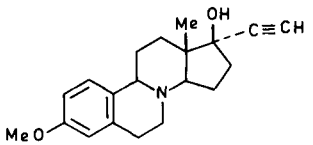
2α-Cyano-17β-hydroxy-4,4,17α-trimethyl-5-androsten-3-one (58) and 17β-hydroxy-4,4,17α-trimethyl-5-androsten[2,3-d]isoxazole and related compounds, which are 3β-hydroxy-5-en steroid oxidoreductase inhibitors, also inhibit *in vitro* 17,20-lyase as manifested by diminished formation of androstenedione and testosterone from 17α-hydroxyprogesterone [400a].

HETEROSTEROIDAL OESTROGENIC, PROGESTATIONAL AND ANTI-FERTILITY AGENTS AND SOME RELATED ASPECTS

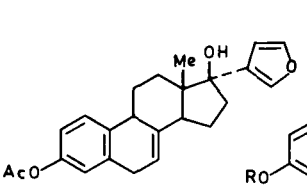
OESTROGENS

Among the nucleo-hetero analogues tested, a particular mention may be made of estrazinol (105) hydrobromide. Its biological effects are similar to ethynylestradiol [401]. Otherwise, the approach of introducing hetero-atoms in estratriene system has not been fruitful. 2-Azaestratriene 3-methyl ether series showed little endocrine activity in a variety of assays [49,50]. ($\pm$)-6-Oxa-8α-estradiol 3-methyl ether [60], 11-oxaestradiol [62], and 16-azaestrone 3-methyl ether [55,56,402] showed very weak oestrogenic activity. 16-Oxaestrone methyl ether was found to be inactive [56]. 2,3-Diaza-A-nor-1,3-androstadien-17β-ol has also been tested and found to be inactive [273].

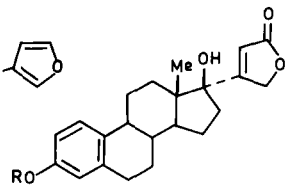
The heterocycles 3-hydroxy-1,3,5(10)-estratrieno[17,16-d]-2′,6′-diamino-



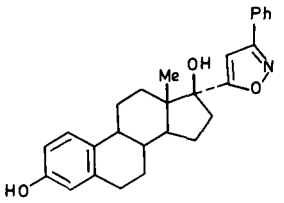
(105)



(106)



(107)



(108)

pyrimidine [317], 17β,2′-epoxy-16β-ethyl-1,3,5(10)-estratrien-3-ol [403], 3-cyclopentyloxy-17β-hydroxy-2′,3′-dihydro-1,3,5(10)-estratrieno[16a,17a-b]furan [404] and 3,17β-dihydroxy-2′,3′-dihydro-1,3,5(10)-estratrieno[16a,17a-b]furan [404] showed weak oestrogenic activity. No oestrogenicity was observed for 17β-hydroxy-17a-methyl-4-androsteno[2,3-d]isoxazole or 17β-hydroxy-17a-methyl-4,6-androstadieno[2,3-d]isoxazole [287,288] which is in contrast to their corresponding [3,2-c]pyrazoles [282]. The pyrazole derivative 17α-cyclopropyl-17β-hydroxy-4-estreno[3,2-c]pyrazole was found to be oestrogenic [405].

17a-(3-Furyl) analogues of oestradiol and dihydroequilin were found to be 4—19 times as potent orally as mestranol in rats (Allen—Doisy test) but were less active in mice [406,407]. The most potent in the series was estrofurate (*106*). Acetylation of the 17-alcohol or replacement of the 3-furyl by a 2-furyl group produced a decrease in activity. The conversion of the furyl group in 17a-(3-furyl)estradiol derivatives into lactones or anhydrides was generally detrimental to oestrogenic activity and only γ-lactones of the type (107) retained the same degree and profile of oestrogenic activity as the starting 17a-(3-furyl)estradiols [408]. The 17a-isoxazolyl steroid (108) had about one-third the oestrogenic activity in the Allen—Doisy assay (p.o. or s.c.) [409]. A series of 7a,8a-epoxyestradiol derivatives with ethynyl, 2- or 3-furyl, or 2-thienyl substituents in the 17a-position

were highly active orally in the Allen—Doisy test in rats, but most of them were only weakly active in the uterotrophic assay in the mouse [410].

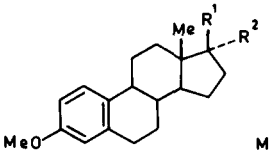
Oestradiol 3-methyl ether derivatives possessing 17α-CH_2X substituents (X = halogeno, pseudohalogeno, N, S and O containing groups) have markedly reduced uterotrophic effect [411,412]. The most active compounds (X = SCN, N_3, $NHCH_3$, $NAcCH_3$) possess about 20 to 50% of the activity of oestriol when given by gavage.

Different estratrienes with hetero substitutions at different positions have been examined. Ethinylestradiol 3-isopropylsulphonate, a depot oestrogen, has given good results on oral administration as 3-mg tablets for inhibition and suppression of lactation in clinical trials [413].

The oestrogenic activity on ovariectomised mice is decreased when 2- or 4-position of oestrone or oestradiol bear substituents [414,415]. Deactivating nitro group causes a greater reduction in oestrogenic activity than the activating groups ($-NH_2$, $-CH_3$) [415].

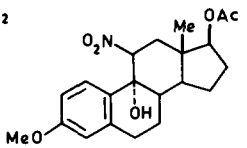
Replacement of 3-oxygen function in oestrogens leads to steep fall in activity. 3-Methanesulphonamido-1,3,5(10)-estratrien-17β-ol [416] and 3-pyrrolidino-1,3,5(10)-estratriene-9α,17β-diol [334] show only very weak uterotrophic activity.

The oestradiol analogue 3-hydroxy-17β-pyrrolidino-1,3,5(10)-estratiene possessed no oestrogenic activity [344]; however, 3-methoxy-17α-methyl-17β-dimethylamino-1,3,5(10)-estratriene (109) is reported to exhibit oestrogenic activity [345]. The 17-thio ethers of the type (110) showed lower activity, when the 17-oxa isosteres (X = Cl,F) have oestrogenic activity 4 times of oestrone when administered orally [417].



(109) $R^1 = -NMe_2$; $R^2 = Me$

(110) $R^1 = -SCH_2CH_2X$; $R^2 = H$



(111)

A series of active 9α-hydroxy-11β-nitro-1,3,5(10)-estratrienes have been reported [418]. In castrated rats, (111) had three times the potency of ethynylestradiol in producing vaginal cornification. It is orally effective despite the fact that it does not possess the 17α-ethynyl function.

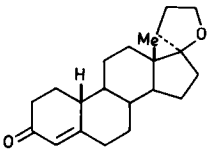
ANTI-OESTROGENS

Several of the compounds tested include ring A fused thiiranes, 17-spiro ethers, nucleo-heterosteroids and 3-oxygen function modifications.

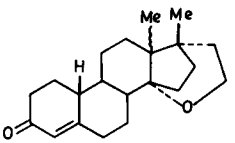
Epitiostanol (2*α*,3*α*-epithio-5*α*-androstan-17*β*-ol, 10275-S) was shown to exert potent and long-acting anti-oestrogenic activity in rodents when administered subcutaneously [325,326,419]. It specifically suppressed the incorporation of oestradiol by the rat uterus and competitively inhibited the binding of oestradiol to its specific receptors from rat uterus cytosol [420].

A derivative corresponding to epitiostanol is mepitiostane (2*α*,3*α*-epithio-5*α*-androstan-17*β*-yl 1-methoxycyclopentyl ether, 10364-S) (92), which was found to antagonise uterine and vaginal responsiveness to exogenous oestrogen when administered orally to immature or ovariectomised mice [421]. These activities were almost equivalent to those of fluoxymesterone, but mepitiostane caused longer-acting inhibition of oestradiol-induced vaginal cornification in spayed mice.

Certain spiro ethers like 19-norspiroxenone (112) [170,422—424] and 14,17-epoxy compounds as (113) [422] are active as anti-oestrogens. The studies on 17-spiro ethers have been extended [425]. Compound (112) and its cyclopropano-6-spiro analogue do not exhibit inherent oestrogenicity [423].



(112)



(113)

16-Azaestrone 3-methyl ether, in addition to being a weak oestrogenic, possesses anti-oestrogenic properties as well [55,402]. 2,3-Diaza-A-nor-1,3-androstadien-17*β*-ol [273] and 17a-oxa-D-homo-5*α*-androstane-3,17-dione [426,427] are inactive as anti-oestrogens. The lactam 17*β*-hydroxy-4-(2-hydroxyethyl)-17*α*-methyl-4-aza-5-androsten-3-one [269] and certain 2,3-diaza-4-methyl-1-one steroids [366] are reported to have some anti-oestrogenic activity [269].

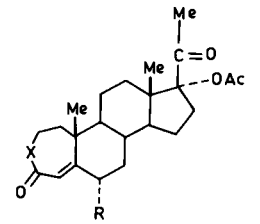
Oestradiol analogues with 3-OH converted to *β*-dialkylaminoethoxy group leads to compounds which are antioestrogenic as tested through *in vitro* binding assay [428]. The cysteine derivative ethyl S-(17*β*-hydroxy-17*α*-methyl-3,5-androstadien-3-yl)-L-cysteinate hydrochloride was tested and found to

be devoid of anti-oestrogenic activity [342]. 2-Cyano-5α-androst-2-en-3β-ol, an active androgen and anabolic steroid, is a potent anti-oestrogen [351].

PROGESTOGENS AND ANTI-PROGESTOGENS

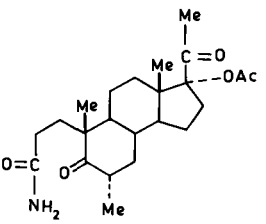
Different types of hetero modifications in the structures of progesterone, 17α-acetoxyprogesterone and ethisterone have been carried out.

As reported in the androgen-anabolic area, the replacement of 2-methylene with oxygen is compatible with the hormonal activity in the progestogen series also. 2-Oxaprogesterone (18) and its 17α-acetoxy analogue are almost as active as progesterone and 17α-acetoxyprogesterone, respectively, in rabbits (Clauberg assay) [58]. However, the 3-oxa-A-homo analogue (114) has been found to be of no interest [46]. The 3-aza-A-homo-6α-methyl analogue (115) has progestational activity (McPhail index 3.0) [429], and the derivative without the 6α-methyl group is only one-tenth in potency. In the ethisterone series, the lactam analogue, 17β-acetoxy-17α-ethynyl-3-aza-A-homo-4a-androsten-4-one was found to lack progestational activity [267].



(114) X = O; R = H

(115) X = NH; R = Me



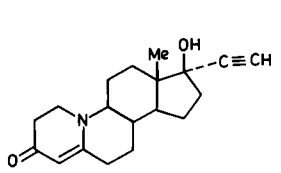
(116)

Interestingly, the ring A seco derivative (116) is reported to be active (McPhail index 3.17 at 5 mg total dose) [268].

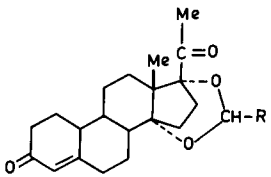
The isosteres with heteroatom in rings B or C have diminished progestational activity. 11-Oxaprogesterone (21) showed subcutaneous activity only one-quarter to one-seventh of that of progesterone [61,62], 11-oxa-17α-acetoxyprogesterone showed only one-eighth of the oral activity as compared with 17α-acetoxyprogesterone [62,430], 11-oxaethisterone has diminished activity [62], and 17α-acetoxy-7-oxaprogesterone did not exhibit a significant level of progestational or anti-progestational activity [431]. (±)-17α-Ethynyl-17β-hydroxy-6-oxa-4-estren-3-one, 6-oxa analogue of norethisterone, has 3% of the activity of progesterone [60]. The 10-

azasteroid (117) was less active than norethisterone but equal to progesterone as an anti-LH compound [432]. 8-Aza-19-nor-progesterone is reported to be inactive [433] or very weakly active [434]. In view of the androgenic activity of 2-thia-A-nor-5α-androstan-17β-ol [270], 2-thia-A-nor-5α-pregnan-20-one was prepared and examined but was found to be inactive [435].

In general, the approach of preparing fused ring heterocycles and derivatives with hetero bridges has led to compounds with diminished activity. 4-Pregneno[2,3-c]furazan-20-one is 0.2 times as active as progesterone [90]. The oral progestational response in rabbits was absent in 17α-cyclopropyl-17β-hydroxy-4-estreno[3,2-c]pyrazole and 17α-cyclopropyl-17β-hydroxy-5(10)-estreno[3,2-c]pyrazole [405], but was present to a moderate degree in 17α-cyclopropyl-17β-hydroxy-4-estreno[2,3-d]isoxazole [405]. 17β-Hydroxy-17α-methyl-4-estreno[2,3-d]isoxazole is mentioned to have oral progestational activity equal to ethisterone [288].



(117)



(118)

17α-Ethynyl-17β-hydroxy-9α,10α-oxido-4-estren-3-one was found to have weak progestational activity with a relative potency one-tenth of norethisterone [334]. 2,3-Epoxy and 2,3-epithio-5α-pregnan-20-ones lacked the activity [436].

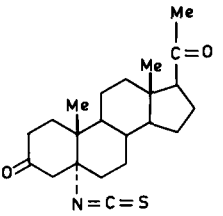
A series of 14α,17α-alkylidenedioxyprogesterone [437] and 14α, 17α-alkylidenedioxy-19-norprogesterone (118) [438] have been prepared and tested for progestational and anticonceptive activities. The optimum activity is in the region of propylidenedioxy compounds. The other progesterone analogue 8-hydroxy-3,20-dioxo-4-pregnen-10-oic acid 19δ-lactone and its 17α-isomer produce only little, if any, progestational action in rabbits [156].

The spiro derivative 17α-hydroxy-3-oxo-23-nor-4,20(22)-choladien-23-oic acid γ-lactone failed to prevent litters being born when fed to mice, and it showed no progestational effect [180]. The spiro ether (112) exhibited progestational activity in immature rabbits when administered by itself [424]; in combination with progesterone especially at high doses, it was

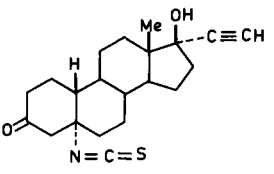
anti-progestational as judged by decrease in endometrial carbonic anhydrase activity.

Different pregnane derivatives carrying hetero substituents at different positions have been tested. 5*α*-Isothiocyanato-5*α*-pregnane-3,20-dione (119) and 17*α*-ethynyl-17*β*-hydroxy-5*α*-isothiocyanatoestran-3-one (120) were approximately equivalent in activity to progesterone and norethisterone, respectively [115]. It may be that these become converted to the corresponding 4-en-3-ones *in vivo* and so exhibit the activity.

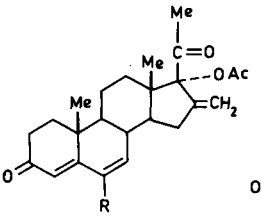
17*α*-Acetoxy-3*β*-pyrrolidino-6-methyl-5-pregnen-20-one showed progestational activity (McPhail grading of 3.1 at 5 mg dose level) [439]. 2*α*-Methylthioprogesterone [112], 20*α*-nitro-4-pregnen-3-one, 20*β*-nitro-4-pregnen-3-one and 3*β*-nitro-5*α*-pregnan-20-one [356] are inactive as progestational agents.



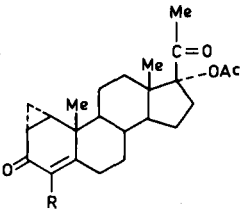
(119)



(120)



(121)



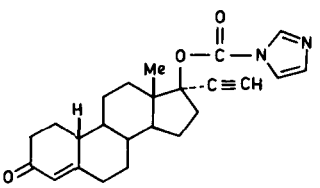
(122)

Chlormadinone acetate, 17*α*-acetoxy-6-chloro-4,6-pregnadiene-3,20-dione, is a highly active progestational agent, as is also its 16-methylene derivative (121,R = Cl). A systematic study [111] relating steric and electronic influences of substituents at position 6 in 6-dehydro-16-methylene-17*α*-acetoxyprogesterones showed 6-azido and 6-thiocyanato compounds to possess moderate progestational activity as compared with methyl and halo relatives. The relative progestational activities (listed in parentheses)

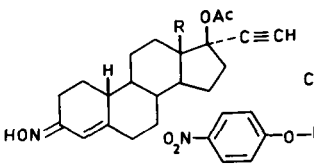
with changing character of R in (121) are: 6-Me (91) > 6-Cl (77) > 6-F (55) > 6-Br (42) > 6-N_3 (20) > 6-SCN (12) > 6-CN (6) > 6-H (1) > 6-$NHCOCH_3$ ($\leqslant 0.1$). In the series, quantitative structure-activity relationships have been examined [440,441]. Certain 4-amino steroidal derivatives (122) have also been reported to be orally active as progestational agents [442].

A mention may be made of some simple derivatives of known progestational agents. 3-Oximino-17α-acetoxy-6α-methyl-4-pregnen-20-one has progestational activity (McPhail index 3.1) [429]. Esters of this oxime have also been studied for progestational activity but no significant alteration in McPhail index was observed [443]. The oral progestational activity of 3-ketals of certain 17-acetoxy and 17-alkyl progesterones [444] is of the order of activity of the parent ketones. In certain cases, activity even appears to be somewhat enhanced. The 3-ethylene ketals of 17α-acetoxyprogesterone and 17α-ethylprogesterone are about 3—4 times as active as the parent ketones (Clauberg assay).

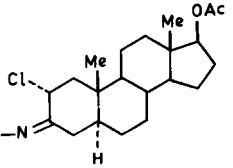
Norethisterone imidazole-1-carboxylate (123) showed twice the progestational activity of (modified Claubert—McPhail assay) and less androgenecity than norethisterone [445]. Lastly, a reference may be made to a study made to prepare anti-progestogens. Acylated derivatives of 17α-hydroxyprogesterone bearing dialkylaminoalkyl moieties were prepared and tested as anti-progestogens [446]. The compounds exhibited low relative binding affinities to progestogen-receptor protein of rabbit uterus, thus making it unlikely that they would be active as anti-progestational agents.



(123)



(124) R = Et
(125) R = Me



(126)

ANTI-FERTILITY ACTIVITY

Different oximes and oximino derivatives have been studied, and norgestimate (124) and norethisterone 3-oxime 17-acetate (125) are of particular interest. Compound (125) was reported to have post-coital contraceptive properties [447]. Norgestimate (124) inhibits ovulation in rats with a potency

of at least 800 times that of (+)-norgestrel [448] and is being studied clinically in combination with ethinylestradiol as an oral contraceptive. The fate of oral administration of (124) in rhesus monkeys [449] and in humans [450,451] has been studied. They become deacetylated in the first instance and then the oximes are hydrolysed to (+)-norgestrel and norethisterone, respectively. Probably norgestimate is not a pro-drug [448]. Norethisterone 3-methoxime has been shown to be more active than norethisterone as an antifertility agent in rats [452].

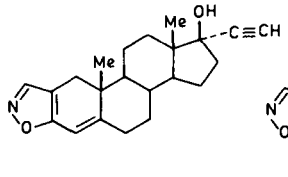
Among the steroidal *O*-aryloximes examined [453] for their ability to interrupt post-implantive gestation in female rats, one of the most active compounds in the series (126) was shown to terminate pregnancy, when orally administered to rats at 2.5 $mg \cdot kg^{-1}$ on days 9—12 of gestation. At this dose level, the compound was devoid of androgenic activity.

A mention may be made of two nucleo-heterosteroids. 11-Oxaprogesterone (21) [61], which showed little progestational activity, has enhanced ovulation inhibitory activity, approximately twice the activity of progesterone, in rabbits in which ovulations were stimulated with copper acetate, but inactive in the rabbit in which ovulation is induced by mating [62]. The lactam, 17β-acetoxy-17α-ethynyl-3-aza-19-nor-A-homo-4a-androsten-4-one, has anti-littering activity six times that of norethisterone [447].

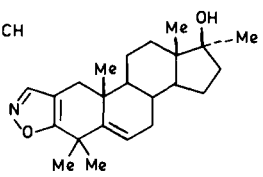
17α-[3-Furyl]-1,3,5(10),7-estratetraene-3,17β-diol 3-acetate (AY-11483) (106), an oral estrogen, is reported to be orally active in preventing pregnancy in rats by interfering with both ovulation and implantation [454]. 3β-Hydroxy-5-androsteno[16,17-c]-5′-methylpyrazole is also reported to have shown antifertility activity in rats [455].

Danazol (Danocrine) (127), an analogue of ethisterone, discovered in 1963 by Sterling—Winthrop workers [288] is a drug of current interest for antifertility and other activities. Certain significant features of the compound may be cited. It inhibits the synthesis and/or release of pituitory gonadotrophins, without exhibiting oestrogenic or progestational activity [456,457]. It has weak, impeded, androgenic activity [457]. In human females, danazol causes suppression of the midcycle surges of LH and FSH, regressive changes in vaginal cytology, amenorrhoea and marked improvement of symptomatology and pathology of endometriosis [458,459]. In males, danazol causes a depression of both circulating androgen and gonadotrophins [460]. It has been suggested that danazol also causes direct inhibition of gonadal steroidogenesis [461]. Danazol is effective in the treatment of endometriosis [458,459,462—467], benign breast disorders [458,468] and precocious puberty [458].

As to antifertility activity of danazol, its effectiveness was first shown



(127)



(128)

on oral administration to rhesus monkeys [469]. At a daily dose of 200 mg or 400 mg per monkey, no pregnancies occurred during the 90-days-treatment period. Danazol prevents ovulation in women at a daily dose of 200 mg, but 100 mg of danazol was incompletely effective in inhibiting ovulation in women [470,471]. There have been pointed out side effects at 200 mg dose level [472], and expanded studies have been suggested for a definitive evaluation of danazol as an oral contraceptive.

Work has been carried on the metabolism of danazol [473,474]. The metabolites isolated include 17-hydroxy-17α-pregn-4-en-20-yn-3-one, 17-hydroxy-2α-(hydroxymethyl)-17α-pregn-4-en-20-yn-3-one, 17-hydroxy-2-(hydroxymethyl)-17α-pregna-1,4-dien-20-yn-3-one, 6β,17-dihydroxy-2α-(hydroxymethyl)-17α-pregn-4-en-20-yn-3-one, and 6β,17-dihydroxy-2-(hydroxymethyl)-17α-pregna-1,4-dien-20-yn-3-one. None of these metabolites exhibited pituitary-inhibiting activity comparable to danazol [474].

The isoxazolosteroid (128) and its esters are claimed to terminate pregnancy in monkeys [475].

3β,3β'-Diselenobis-5-androsten-17-one consistently inhibited the endometrial proliferation produced by progesterone in the rabbit [398]. Despite this, the disteroid was not capable of inhibiting ovulation and implantation in the rat.

20,25-Diazacholesterol (azacosterol, as its hydrochloride, Ornitrol) is of interest as a chemosterilant in the control of pest pigeon population [476]. Ingestion of this compound by pigeons causes inhibition of egg production and temporary sterility. It is suggested that inhibition of cholesterol side chain cleavage and an accompanying decrease in steroid hormones production may play a role in the anti-fertility action of the drug [477].

Considering the anti-fertility action of heterosteroids in males, again a reference may be made to danazol (127). It was studied in normal adult males [458,460,477—479]. The most effective treatment of suppression of spermatogenesis was with danazol 600 mg orally daily and testosterone enanthate 200 mg intramuscularly monthly [479]. Within 12 weeks, the majority of sperm counts of volunteers fell to below $5 \times 10^6 ml.^{-1}$ The effect on spermatogenesis is temporary, and after treatment spermato-

genesis recovers completely to pre-treatment values. Addition of a testosterone derivative helps in enhancing the depressive effect of danazol upon spermatogenesis and removes the problem of impaired libido [478]. It is suggested that danazol acts by inhibiting spermatogenesis at the spermatocyte-spermatid level rather than causing premature sloughing of the germinal epithelium [479].

The anti-androgen cyproterone acetate (102) has been the subject of study for its action on reproductive functions in human males [480,481]. Daily administration of 5 or 10 mg of cyproterone acetate to normal volunteers over a period of 20 weeks caused a gradual decrease in the count and motility of spermatozoa, concurrently with an increase in the percentage of non-motile as well as abnormal and immature sperms [481]. It is claimed that at low dose therapy, libido and potentia are not adversely affected.

19-Norspiroxenone (112) which is a potent anti-oestrogen has been shown to cause striking arrest of spermatogenesis in male rats [482,483]. Some bis-dichloroacetamido steroids when tested in Holtzman rats caused reversible inhibition of spermatogenesis [483a].

CATATOXIC HETEROSTEROIDS

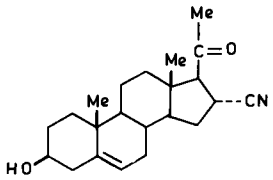
The nonspecific protective action of some steroids against drug intoxication is mediated either through body's increased tolerance which may be a result of reduced tissue sensitivity to the drugs (syntoxic effect) or the accelerated biotransformation of the toxicant (catatoxic effect), often achieved via the induction of microsomal drug metabolizing enzymes, mostly in the liver [484—486]. The syntoxic effects are virtually limited to glucocorticoids.

In an effort to explore the relationships between catatoxic potency and chemical structure, a large number of steroid compounds including several hetero analogues were tested for their ability to protect the rats against usually fatal intoxication with indomethacin or digitoxin [484,485]. It appears that catatoxic activity is very widespread among steroids. Catatoxic effect can manifest itself independently of all classic hormonal actions, although it is frequently associated with anabolic, anti-mineralocorticoid, or glucocorticoid properties. Oxandrolone (81) is an active catatoxic agent [484—487], but pregnenolone-16α-carbonitrile and spironolactone are more potent.

PREGNENOLONE-16α-CARBONITRILE (PCN)

Some of the most potent catatoxic steroids are carbonitriles, the 16α-position of the —CN group being particularly advantageous. Pregnenolone-

16α-carbonitrile (3β-hydroxy-20-oxo-5-pregnene-16α-carbonitrile, PCN, 129), synthesised in 1958 [488,489], and first studied by Selye [484], is the most potent catatoxic steroid so far tested. PCN was also shown to be potent in reducing the toxic manifestations of various compounds [490—496]. The esters of PCN are more active than the parent compound, probably due to their rapid absorption [490]. PCN and other catatoxic steroids have been shown to inhibit the effects of steroid anaesthetics [497]. PCN induces hepatic microsomal [498—503] as well extramicrosomal drug metabolising enzymes, such as phosphoprotein phosphate [504]. These findings have been confirmed by several workers [505—509].



(129)

In rats PCN is potent inducer of ethylmorphine demethylase and aniline hydroxylase to a similar extent in both regenerating and intact liver [510]. Using inhibitors of protein synthesis, it has also been shown that the induction process in regenerating liver may be inhibited at transcriptional and at translation levels. However, the degree of induction of the same enzyme by spironolactone was much less in regenerating liver than in normal liver. Spironolactone may induce drug-metabolising enzyme synthesis through different mechanisms than PCN; spironolactone, unlike PCN, fails to increase cytochrome P-450 content in the liver [511]. It has been shown that on PCN treatment, the onset of increase in aniline hydroxylase activity and cytochrome P-450 content is slower in male than in female rats [512].

It is of interest to note that distinct from the cytochrome P-450 components synthesised in response to barbiturates or polycyclic hydrocarbons, PCN induces the synthesis of a unique cytochrome P-450 peptide in hepatic microsomes of male rats [513]. This suggests a basis for the differential spectral and enzymatic properties resulting from PCN induction. PCN increased bile flow but did not alter bile salt excretion, indicating that PCN like phenobarbitone and spironolactone increased bile salts independent of bile formation [514].

PCN in rats increased the 6β-hydroxylation of lithocholic acid about

twice and the 7*a*-hydroxylation three to four times [515]. The ability of PCN to prevent lithocholic acid-induced cholelithiasis [516] may be to an extent due to the inducing effect of PCN on the microsomal hydroxylations of lithocholic acid. As judged by *in vivo* experiments in rats, PCN does not modify the steady state steroid-dependent physiologic functions [517]. Biochemical studies are required to elucidate the exact mechanism(s) by which sex steroids ensure pregnancy evolution despite microsomal enzyme inducer such as PCN.

PCN appears to be well tolerated in man [518]. Since it has no known pharmacologic actions other than enzyme induction, it should be suitable in acute and chronic drug overdosage (*e.g.* with digitoxin, indomethacin), non-haemolytic, non-obstructive jaundice, kernicterus and long term exposure to pesticides. The protective effect of PCN is probably mediated through induction or activation of hepatic drug-metabolising enzymes, or both.

SPIRONOLACTONE AS A CATATOXIC AGENT

Pretreatment with spironolactone (60) abolishes the anesthetic effect of progesterone, deoxycorticosterone and hydroxydione and prevents digitoxin intoxication in the rat [519]. Spironolactone also diminishes the toxicity of pentobarbital, indomethacin, diphenylhydantoin, 7,12-dimethylbenzo [*a*] anthracene and of many other structurally unrelated compounds [484,520]. A limited protection against the toxicity of parathion by preadministered spironolactone has been observed [521]. A study on rat suggests that spironolactone decreases the toxicity of indomethacin by increasing its biotransformation and excretion into the bile [522], as with phenobarbitone and PCN. In the same experiments, 3-methylcholanthrene was without effect. It may be related to the different forms of cytochrome P-450 produced in response to the various inducers [523].

The ability of spironolactone to reduce the toxicity of cardiac glycosides in mice and rats is independent of its potassium-sparing effect [487,524—527]. Through experiments using [^{3}H]digitoxin [528—530] and [^{3}H]digoxin [531], it has been suggested that the protective effect of spironolactone on cardiac glycosides toxicity is caused by increased metabolism of the glycosides and increased fecal excretion of the metabolites. The metabolism is increased by induction of hepatic microsomal enzyme activity [499,531—533].

The increased fecal elimination is thought to reflect primarily increased biliary elimination under the influence of spironolactone, as also with PCN [534—536]. Spironolactone or PCN cause an increase in the hepatic bilirubin metabolism by stimulating the activity of the bilirubin glucuronyltransferase [536].

It has been shown in rats that the enhanced elimination of 4'''-methyldigoxin after pretreatment with spironolactone is the result not only of an enhanced metabolism but also of an increased hepatic uptake and biliary excretion of the drug and its metabolites [537]. However, in contrast to experiments in rats, spironolactone exerts only little influence on pharmacokinetics of 4'''-methyldigoxin in man [538]. The latter findings are consistent with the clinical observations where no protective effect of spironolactone in the treatment of digitalis intoxication could be demonstrated [539,540]. In a recent study carried out in dogs, it has been indicated that spironolactone acts to inhibit the toxicity of digoxin selectively while not altering its inotropic action [541].

The broad resistance-increasing of spironolactone is explained by its ability to increase various NADPH-dependent enzymatic reactions in liver microsomes and that enhancing the degradation of many drugs and their elimination from the body [542—546]. This presumption is supported by *in vitro* studies on liver microsomes of spironolactone pretreated rats in which cytochrome *c* reductase activity was enhanced and cytochrome P-450 content was diminished [532,547]. It is reported that the protective action of spironolactone is completely suppressed by ribonucleic acid- or protein-synthesis inhibitors such as actinomycin D, puromycin aminonucleoside and cycloheximide [548].

Combined administration of spironolactone and carbon tetrachloride was found to potentiate the toxic action of carbon tetrachloride and was accompanied by a decrease in the total activity of all the enzymes in the liver tissues and an increase in free β-glucuronidase and acid phosphatase activity in the liver tissue and blood serum [549]. Preliminary administration of spironolactone prevented the decrease in activity of the enzymes but did not affect the stability of the lysosomes during carbon tetrachloride poisoning.

Treatment with spironolactone protected the acute toxicity not only caused by mercuric chloride [550] but also caused by different organomercurials including methoxyethylmercury chloride in rats [551]. Here the catatoxic effect may be through binding between the thioacetyl group of spironolactone and the mercury ion or the protection may be due to promoting the synthesis of SH-containing compounds through a nonspecific enzyme induction by the hepatic microsomes. It may be mentioned that there has been observed *in vitro* enhancement of erythrocyte mercury uptake by spironolactone, evidently through complexation with 7-thioacetate group, as canrenone (78) failed to increase mercury uptake [552]. It has also been postulated [553] that a possible mechanism for the protective effect of

spironolactone against mercury poisoning is the change of mercury distribution in the body tissues, particularly the decrease of mercury concentration in the kidney. A single dose of spironolactone administered shortly before mercuric chloride injection can produce enhanced plasma clearance, erythrocyte and hepatic uptake and biliary clearance [554]. The effect of spironolactone on biliary excretion of mercury could be limited by the level of 'mercury available' in the organism and might be determined by direct interaction of mercury molecule with spironolactone (or its metabolite) [555]. In similar fashion to mercury, copper appears to be more rapidly cleared from plasma, taken up by liver, and excreted into bile under the influence of spironolactone [556]. Erythrocyte copper levels, however, are not increased. Biliary excretion of mercury in rats, but not of cadmium, zinc and cerium, on spironolactone pretreatment has been shown [557]; however, other workers report no significant increase in mercury excretion in rats [553,558]. The discrepency in the observations may be due to the difference in the mercury dose used [559]. Pretreatment with spironolactone also decreased the acute lethal toxicity induced by cerium chloride [560,561].

CARDIAC AND ANTI-HYPERTENSIVE HETEROSTEROIDS

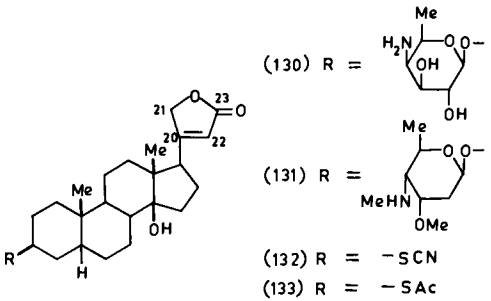
CARDENOLIDES AND SOME RELATED ASPECTS

The digitalis glycosides continue to be important in the treatment of congestive heart failure. Rigorous research efforts are being made to devise a more useful drug with less danger of cardiac toxicity. Two recent reviews on cardenolides and allied literature are of interest [562,563].

The cardenolide structure has been modified in different ways including changes of the butenolide ring attached to position 17. Alterations have also been made in the sugar moieties attached to position 3.

A semi-synthetic aminosugar glycoside, 3β-*O*-(4-amino-4,6-dideoxy-β-D-galactopyranosyl)digitoxigenin (ASI-222) (130), patterned after naturally-occurring 4-aminosugar cardenolide [564,565], has been shown to have a greater therapeutic index than ouabain in vagotomized dogs [566]. It is three times more potent than digoxin in increasing cardiac output, minute cardiac work and stroke work in the dog heart—lung preparation [567], and produces an earlier and greater increase in contractile force and dP/dt than digoxin in equimolar doses [568].

Mitiphylline (131), a naturally-occurring aminosugar cardiac glycoside [565], with a structure similar to ASI-222, has positive inotropic effect and is more potent than digitalin on rabbit heart [569].

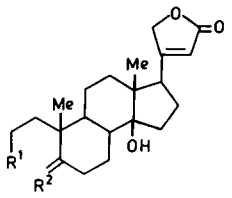


Cardiac glycosides interact specifically with high affinity at the cardiotonic steroid binding site of Na^+,K^+- ATPase. It is suggested that this site may be a pharmacological receptor for these drugs [570]. Attempts have been made to affinity-label this receptor. Strophanthidin-3-bromoacetate (SBA) [571] and digoxigenin-3,12-dibromoacetate (DDB) [572] have been examined. It was seen that interaction of SBA was readily reversible [573], whereas DDB irreversibly inhibited specific [^{3}H]ouabain binding to Na^+,K^+-ATPase [572]. But this inhibition apparently did not involve cardiotonic steroid binding sites of Na^+,K^+-ATPase. Apart from these alkylating derivatives of cardiac aglycones, tests have been made with 3-azidoacetylstrophanthidin (AAS), a photochemical analogue of strophanthidin [574]. A substantial increment in irreversible inhibition of Na^+,K^+-ATPase by AAS was observed in the presence of UV light plus acetylphosphate.

The oxygen function at position 3 in cardenolides does not seem to be required for cardiotonic activity. 3-Deoxydigitoxigenin has shown cardiotonic activity comparable with that of digitoxigenin in the isolated frog heart [575,576] and in a Na^+,K^+-ATPase inhibition assay [577]. 3-Deoxydigitoxigenin analogues with variations at other positions have been studied [578,579]. The 3*α*- and 3*β*-amino-3-deoxydigitoxigenins have been prepared [579], but the biological activity does not appear to have been described. The 3*β*-thiocyanato (132) [580] and 3*β*-thioacetyl (133) [581] derivatives have been shown to exhibit cardiotonic effect in animal experiments and inhibit Na^+,K^+-ATPase.

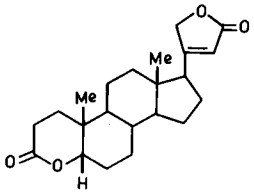
Intact ring A in cardenolides may not be necessary for cardiotonic activity. The 3,5-seco-4-norcardenolides (134) and (135) produced definite cardiotonic activity on frog hearts and guinea-pig atria [582,583]. The 4-oxacardenolide (136) was more potent than its 5*α*-isomer. The heterocyclic cardenolide analogues other than 4-oxacardenolide may also be expected to possess the cardiotonic activity.

The butenolide ring of the cardenolides has been varied in different ways.



(134) R^1 = -COOMe
R^2 = O

(135) R^1 = $-CH_2OH$
R^2 = 



(136)

There are known cardenolides in which 22-position has been substituted with halogeno, alkyl and alkoxy groups [584]. The contraction-promoting activity is increased in the fluoro derivative and decreased in methoxy analogue. Activity has also been claimed in derivatives possessing —F, $-N_3$,$-ONO_2$ substituents at position 21 [585].

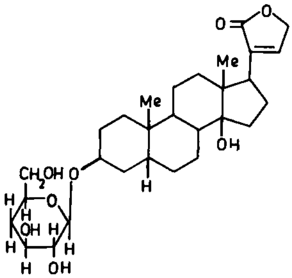
Recently, chemical properties [586] and biological activity [587—589] of actodigin (AY-22,241) (137) have been studied. It is a new semisynthetic cardiac glycoside, which differs from naturally occurring glycosides in terms of the attachment of the lactone ring to the steroid nucleus in the aglycone part. This glycoside has an extremely rapid onset and brief duration of action. It is reported to have positive inotropic effects in doses which are a smaller fraction of the lethal dose than is the case for ouabain or dihydroouabain [588].

Certain other isomeric cardenolide analogues in which the lactone ring is substituted at the γ-carbon instead of the β- or α-carbon found respectively, in the natural cardenolides or as in isomer (137), have been prepared but found to be inactive [590,591].

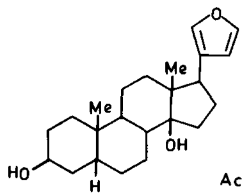
17β-(3-Furyl)steroids as (138) exhibit cardiotonic activity comparable with cardenolides [592]. The positive inotropic effect of 17β-(3-furyl)-5β,14β-androstane-3β,14,16β-triol 3-bisdigitoxoside (FGBD) and the 3-tridigitoxoside (FGTD) has been demonstrated in guinea-pig, rabbit and frog hearts [593].

The lactam analogue (139) [594,595] corresponding to isodigitoxigenin lacks cardiotonic activity but it inhibits the early inotropic response of acetyldigitoxigenin on isolated guinea-pig atria, may be by inhibiting the mechanism responsible for transport of cardiotonic steroids across the cell membrane.

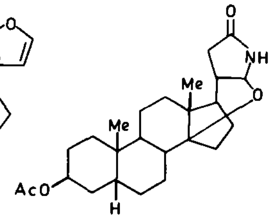
It was claimed that compounds of 17β-(2-substituted-4-thiazolyl)-4-andro-



(137)



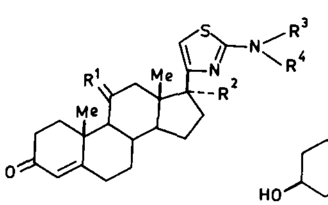
(138)



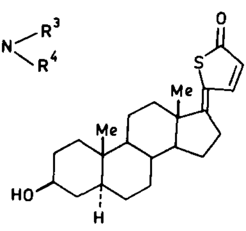
(139)

sten-3-one series possess activity like cardiac glycosides [596], but no such activity was found in 17β-(2-amino-4-thiazolyl)-4-androsten-3-one derivatives [597]. However, there have been prepared a series of 17β-(*N*-substituted-2-amino-4-thiazolyl)-4-androsten-3-one compounds of general structure (140) and the related 3β,17α-diol series and hydrogen succinates, and these were reported to possess cardiac aglycone-like properties in pigeon method and Engelmann's isolated frog heart test [598,599]. The analogue 17β-(2-amino-4-thiazolyl)-5β,14β-androstane-3β,14-diol 3-acetate hydrochloride showed no inotropic activity (rabbit atria test) [600].

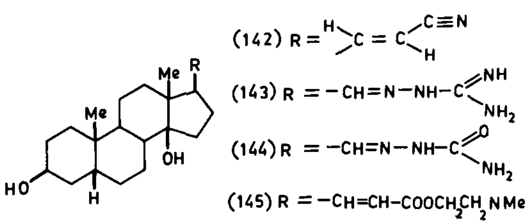
Synthesis of 17-(5-isoxazolyl)- and 17-(3-pyrazolyl)-steroid derivatives has been carried out [601,602] but these have been reported to be inactive [562]. *N*-(Steroid-17-yl)maleimide entities have been prepared [603], but no biological data are provided.



(140)



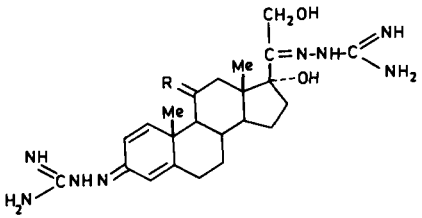
(141)



Thiobutenolides 3β-hydroxy-γ-mercapto-5α-androstane-$\Delta^{17,\gamma}$-crotonic acid γ-lactone (141) and 3-hydroxy-γ-mercapto-1,3,5(10)-estratriene-$\Delta^{17,\gamma}$-crotonic acid γ-lactone, both mixtures of geometric isomers, increased contractile force, heart rate, and blood pressure in dogs [604]. It was concluded that the activity was probably due to release of endogenous norepinephrine from cardiac sympathetic nerve endings.

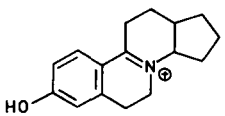
Analogues of cardenolides have been prepared in which the butenolide ring at position 17 has been replaced with a series of open-chain moieties. For example, the α,β-unsaturated nitrile (142) possessed a strong stimulant action on the atria with a potency approximately two-third and the guanylhydrazone (143) one-fifth as potent as compared with digitoxigenin, and the semicarbazone (144) was inactive [605]. Some new biologically active 17β-formylguanylhydrazones have recently been prepared [605a].

While correlating Na^+,K^+-ATPase inhibition and ion transport *in vitro* with inotropic activity and toxicity in dogs, it was seen that basic esters, like (145), are about as active as the corresponding cardenolides [606]. The *in vitro* tests can be used satisfactorily to predict inotropic activity, but not toxicity or therapeutic ratio. Certain 21-haloacetoxy-20-oxopregnanes showed appreciable cat cardiotoxicity, but tests for cardiotonic activity were negative [600].



(146) R = O

(147) R = β −OH, α−H



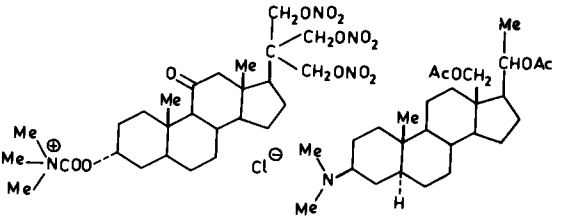
(148)

Prednisone bisguanylhydrazone (146) and prednisolone bisguanylhydrazone (147) were reported in 1964 [607,608] as a new class with digitalis-like activity. A comprehensive paper on the topic has been published by Schütz, Meyer and Krätzer [609], and the structure-activity relationship has been reviewed. Apart from bisguanylhydrazones of steroids, those of some nonsteroid molecules are also active. The pharmacokinetic properties of the steroidal guanylhydrazones have been investigated [610], and the pharmacology of bisguanylhydrazones in laboratory animals and in humans has been reviewed [611]. Triamcinolone-3,20-bisguanylhydrazone has been studied in humans. It is effective but its duration of action is too brief.

Lastly, a mention may be made of a series of 8-azasteroid related analogues possessing cardiovascular, and in particular anti-shock properties [612]. No structure-activity relationship was evident. The analogues showing inotropic and anti-shock activity inhibited metal ion-stimulated heart membrane ATPase, just as did ouabain and the Erythrophleum alkaloids, whereas the biologically inactive compounds had no effect on the enzyme [613]. A derivative of particular interest was quindonium bromide (148, cis). Its dominant cardiovascular effects are a positive inotropism, bradycardia and vasodilation [614]. In addition it shows an indirect cholinergic action on the gut. A study has been made of the effect of quindonium bromide on dogs which have been exposed to lethal haemorrhage shock [615]. Prophylactic administration of quindonium (10 $mg \cdot kg^{-1}$) 15 min before haemorrhage produced a significant increase in survival rate of dogs exposed to haemorrhage stress. Glucuronide is the major metabolite of quindonium bromide in mice, rats, rabbits and dogs [616].

CORONARY DILATORS

There have been designed bis- and trisnitratomethyl steroids and related derivatives, for example (149); in a way, these are analogous to glyceryl trinitrate and these have proved to be potent coronary vasodilatory agents [617—623]. Simple ester testosterone nitrate also prevents coronary spasms in rabbits [122]. Certain steroidal amines such as 3α-acetoxy-11-oxo-20-dimethylaminoethyl-21-dimethylaminopregnane [624] and (150) [625] have also shown coronary dilatory action.



(149) (150)

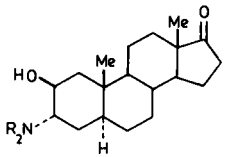
ORG 6001—AN ANTI-DYSRHYTHMIC AMINOSTEROID

The topic of arrhythmias and anti-arrhythmic drugs has been reviewed recently [626,627]. Here a note is taken of the anti-arrythmic non-hor-

monal aminosteroid Org 6001, hydrochloride of (151), developed by the Organon [628]. It does not stimulate central nervous system. It is neither a potent local anaesthetic in the mouse *in vivo* nor an β-adrenoceptor blocker. It has been shown that Org 6001 is effective when administered both intravenously and orally in suppressing the early arrhythmias induced by acute one-stage coronary artery ligation in anaesthetized dogs [629]. It was also observed that some of the metabolic indices of myocardial ischaemia (*e.g.* CO_2 and lactate production, K^+ efflux) were reduced in dogs treated with Org 6001. A follow-up study suggests that the effect of the drug of reducing the ST-segment elevation produced by coronary occlusion in the dog is related either to a reduction in the extent and degree of myocardial ischaemia or to prevention of K^+ egress from ischaemic cells [630].

Org 6001 has also effective anti-arrythmic properties in controlling ouabain-induced ventricular tachycardia in dogs with acceptable cardio-depressant actions [631]. The electrocardiographic alterations observed in the anaesthetized pig when doses in excess of 20 $mg \cdot kg^{-1}$ of Org 6001 were administered, prove that this compound also possesses significant electrophysiological properties [632]. However, as an anti-arrythmic in dogs, it is effective at doses less than 20 $mg \cdot kg^{-1}$.

A study on the rabbit cardiac muscle shows that Org 6001 is an anti-arrythmic drug of the highest class (local anaesthetic type), and within this group it is of sub-class more closely related to lignocaine than to quinidine [633]. It has been reported that the effective refractory period (ERP) in human ventricular muscle and the ERP/action potential duration ratio in sheep Purkinje tissue was not significantly altered by the drug [634].



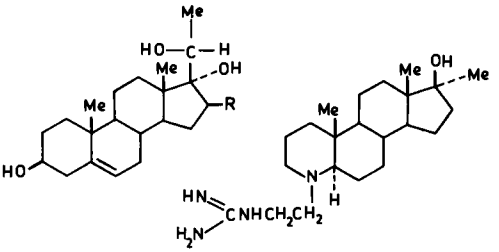
(151) R = H
(152) R = Me

The dimethyl analogue Org NA13 (152), which is potent local anaesthetic [635], is discussed later. Experimental arrhythmias induced in mice by chloroform, in rats by aconitine and in dogs by coronary artery ligation were corrected by Org NA13 at doses from 10 to 50 $mg \cdot kg^{-1}$ intravenously.

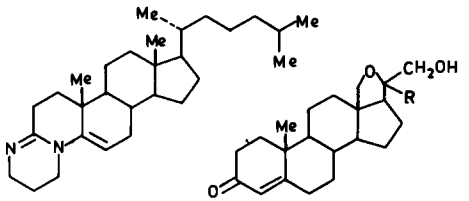
ANTI-HYPERTENSIVE AGENTS

The veratrum alkaloids are well known for their anti-hypertensive properties [636]. An alkaloid, chemically 20α-amino-5-pregnen-3β-yl β-D-glucopyranoside, isolated from roots of Conopharyngia pachysiphon, has shown anti-hypertensive activity on intravenous administration [637]. Some synthetic analogues, e.g., 20-(5-methylpiperid-2-yl)-5-pregnene-3β,20-diol, are also reported to be active [637a].

Hypotensive activity, in pentobarbitone-anaesthetized dogs with cannulated femoral arteries, was shown by certain 16β-alkylamino-5-pregnene-3β, 17α,20β-triols (153) [638]. The analogue (154) having part of the structure corresponding to guanethidine is reported to have weak anti-hypertensive activity in hypertensive rats [639].



(153) R = -NHEt = -NHPr = -N(Et)Me (154)



(155) (156) R=OH (157) R=H

17β-(Imidazol-4[5]-yl)-5-androsten-3β-ol and 17α-hydroxy-17-(imidazol-4[5]-yl)-4-androsten-3-one were found active when tested for depressor activity in metacorticoid hypertensive rats [640]. 3β-Hydroxy-16α-piperidino-5-pregnen-20-one has shown hypotensive activity in dogs at a dose of 1—2 mg·kg^{-1} [641].

The compound (155) possessed hypotensive activity when administered subcutaneously to Sprague—Dawley rats. The effect on blood pressure was much greater after administration for several days and in deoxycorticosterone acetate-hypertensive than in normotensive rats [642].

18-Hydroxydeoxycorticosterone (156) has been suggested to be a causative agent in hypertension [643—645]. The analogue (157) caused in genetic hypertensive rats and in rats suffering from adrenal regeneration hypertension a decrease in blood pressure within 3 h after i.p. injection [646].

ANTI-LIPEMIC ACTIVITY AND HETEROSTEROIDS

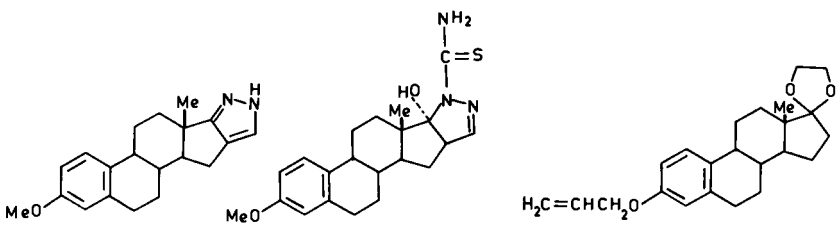
ESTRATRIENE DERIVATIVES

Different hetero modifications of steroidal oestrogens have been tried with the objective of preparing compounds having lipid-lowering properties but lacking feminizing effects.

Some nucleo-heterosteroids have been tested. 2-Azaestratrienes were found to be hormonally inactive but they showed moderate hypocholesterolemic activity *in vivo* [49]. (±)-6-Oxa-8α-estradiol methyl ether had 60% of the activity of estradiol in a rat blood—lipid depressing test but possessed negligible oestrogenic activity [60]. 16-Aza-estrone methyl ether, a very weak oestrogen, exhibited hypocholesterolemic activity in adult male rats in 2 $mg \cdot kg^{-1}$ dose [56].

Certain estratrieno[17,16-c]pyrazoles have been prepared [647,648]. The pyrazole (158) showed hypocholesterolemic activity approximately equal to that shown by oestradiol, while the oestrogenic activity was 0.0001 times that of oestradiol [648]. The analogue (159) is claimed to have still better separation of the activities [649]. The heterocycle 1,3,5(10)-estratrieno [17, 16-d]-2′,6′-diaminopyrimidin-3-ol exhibited no antilipemic effect [317] and 1,3,5(10)-estratrieno[17,16-c]-2′-methylfuran-3-ol showed both weak oestrogenic and hypocholesterolemic activities at 10 $mg \cdot kg^{-1}$ dose in rats [650]. Hypolipemic activity of 1α,2α-oxido-4,6-androstadiene-3, 17-dione in rats and rabbits has also been reported [651].

Knowing that estrone methyl ether, ethylene ketal [652] and estrone hemithioethylene ketal [653] possess low oestrogenic activity, the Wyeth Laboratories team [654] examined a number of estrone ketals for antilipemic and feminizing activities. The alkyl ether ketal (160), having the highest anti-lipemic activity with best separation of activities among the



(158) (159) (160)

ethylene ketals studied, was the compound of interest. The hemithio- and dithioketals had lower activity than the corresponding ketals.

In lipodiatic assays, 3-methoxy-2-sulphamoyl-1,3,5(10)-estratrien-17β-yl acetate, inactive as an oestrogen, showed minimal activity in lowering serum cholesterol level [655].

Gonane derivatives, as 3-(2-pyrrolidinoethoxy)-17,17-dimethyl-1,3,5(10),8,11,13-gonahexaene, showed significant cholesterol-lowering activity in rats, but they produced undesirable effects [656]. 16-Haloestrone derivatives have been of interest as anti-lipemic agents [657,658]. 3-Deoxy-16-halo-1,3,5(10)-estratrienes also show a high lipodiatic/oestrogenic ratio [659].

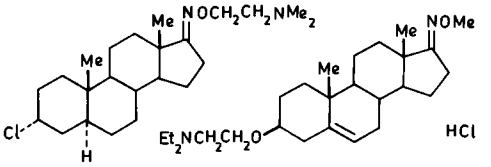
ANDROSTANE ANALOGUES

Several androstane derivatives, including certain potent heterosteroidal anabolic androgens, have been found to be active.

Oxandrolone (81) is effective in lowering the plasma glyceride level in patients with different types of hyperlipoproteinemia [660—663] and has been used in Brazil for the treatment of hyperlipoproteinemia. It has been demonstrated that oxandrolone lowers the plasma cholesterol levels in hyperlipidemic retired-breeder rats [664]. Unlike a number of other hypolipidemic drugs, oxandrolone does not induce a marked proliferation of hepatic microbodies [665]. It has also been reported that oxandrolone had a slight hepatomegalic effect when fed to rats as 0.15% of the diet, without an effect on serum or liver lipids [666].

Heterosteroidal anabolics having heterocyclic ring systems fused to ring A have been tested for anti-lipemic activity. 7α-Ethylthio-17β-hydroxy-17α-methyl-5α-androstano[3,2-c]pyrazole (PS-179) [667], 7α-ethylthio analogue of stanozolol (85), furazabol (89) [668] and 2α,3α-epithio-5α-androstan-17β-yl 1-methoxycyclopentyl ether (10364-S) (92) [669] have been shown to have potent hypolipidemic activity in animal experiments.

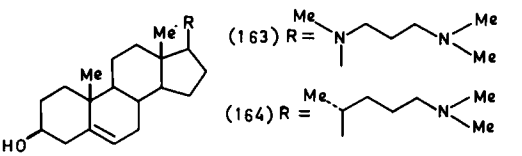
Steroids with a dimethylaminoethoxyimino side chain at C-17 have shown potent hypocholesterolemic activity in rats [670]. Among the compounds tested, the 3*α*-chloro-5*α*-androstane derivative (161) exhibited the highest potency. Certain 3*β*-(2-dialkylaminoethoxy)-substituted steroids have been examined for their hypocholesterolemic effect [671,672]. They apparently inhibit the coversion of desmosteral to cholesterol. Some members, for example, compound (162) possessed an activity at least twenty times that of triparanol in rats [672].



(161) (162)

ANTI-LIPEMIC AND SOME OTHER ASPECTS OF AZASTEROLS

The observed negative feed-back control of cholesterogenesis in animals [673—675] and man [676] led Searle chemists [677,678] to prepare diaza analogues of cholesterol, and they obtain in 20,25-diazacholesterol (163) an active inhibitor of cholesterol synthesis in animals [678—680] and in humans [681]. A study in the structure modification revealed that the mono-aza derivative 25-azacholesterol (164) was ten times more active than (163) [682]. The azasterols inhibited desmosterol reductase, but the overall reduction in total sterols in treated rats was considered to be an indication for a site of action prior to the cyclization of squalene.



Different oxa and oxa-aza side chain analogues of cholesterol have been prepared [683,684]. It has been demonstrated [684] that 25- and 24-aza cholesterols and related compounds are highly potent hypocholesterolemic agents, regardless whether C-20 or C-22 are present as carbon atoms or whether they are replaced by oxygen or nitrogen. Further studies in rings A

and B modified derivatives [685] and 17*a*-epimer stereoisomer [686] cholesterol and C-20 epimer [687] of 22,25-diazacholesterol yielded less active compounds. 25-Azadihydrolanosterol has been found to be at least as active as 20,25-diazacholesterol [688]. Weak oestrogenic property of ring A aromatic diazasterols 3-methoxy-20,25-diaza-19-nor-1,3,5(10)-cholestatriene [685] and 22,25-diaza-19-nor-1,3,5(10)-cholestatrien-3-ol [689] is possibly not entirely responsible for their hypocholesterolemic activity. Bile acid analogue, ethyl 3*β*-hydroxy-20-aza-21-nor-5-cholen-24-oate, is much less active than 20,25-diazacholesterol [690].

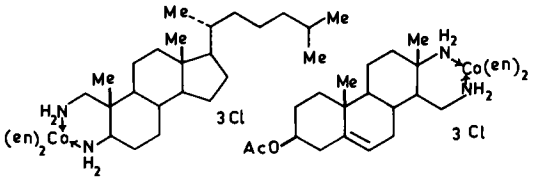
It is of interest to note that acyclic amines such as *N,N*-dimethyl-3,7,11-trimethyldodecanamine were found to lower plasma sterols in rats as did 25-aza-5*a*-cholestane [691]. 6-Amino derivatives of stigmastanol and cholestanol induced hypocholesterolemia in rats [692]. It is reported that (22R)-22-aminocholesterol does not affect serum or liver cholesterol levels but significantly inhibits the activity of liver enzymes concerned with lipogenesis and cholesterol hydroxylation [693].

Structural modification of 5*a*-cholestane-3*β*,5*a*,6*β*-triol, a substance with marked hypocholesterolemic activity in experimental animals, has led to the synthesis and *in vitro* biological evaluation of the 3*β*-, 3*a*-, 5*a*- and 6*β*-monoamino and 3*β*,6*β*-diamino analogues of the triol, and selected azido and oximino intermediates [694]. Study has been made of their inhibitory action on [2-^{14}C]acetate and [2-^{14}C]mevalonate incorporation into unsaponifiable products catalyzed by a rat liver homogenate preparation and their inhibitory or stimulatory action on two semipurified liver enzymes, Δ^7-sterol Δ^5-dehydrogenase and $\Delta^{5,7}$-sterol Δ^7-reductase. There seems to be direct effect on the microsomal enzyme and alteration of the function of sterol carrier protein required for full activity of the enzyme. 6-(Dimethylaminomethyl)-5*α*-cholestane-3*β*,5*α*,6*β*-triol hydrochloride is also claimed to be active as hypolipemic agent [695].

There is interest in aza analogues of cholesterol for their inhibitory effect on cholesterol side-chain cleavage. They have been shown to be inhibitors of conversion of cholesterol to pregnenolone (using mitochondrial fraction of bovine adrenal cortex as the enzyme source). It was observed that with (22R)-22-aminocholesterol and the oxime of 22-oxocholesterol virtually complete inhibition occurred at equimolar cholesterol and inhibitor concentrations (3.3 μM) [696]. The (22S)-amino stereoisomer was somewhat less active. These derivatives are more active than 22-azacholesterol [477] which at equimolar concentrations of inhibitor and substrate of 3 μM exhibited an inhibition of approximately 60%; 22-azacholesterol was a better inhibitor than 20 or 25-azacholesterol [477,697].

A mention may be made to the effect that 20,25-diazacholesterol (163) produces myotonia in man [698] and in animals [699,700], as do some other azasterols [700]. It has been suggested that the accumulation of desmosterol in plasma and in sarcolemma so alters normal permeability of the muscle membrane that myotonia develops [701—704]. It appears that combination of a decreased Cl^- permeability and increased Na^+ gradient induces myotonia [705]. It may be that both these phenomena are related to the changed sterol content of the sarcolemma. It has been stressed that while the 20,25-diazacholesterol may be good as a model of myotonia, and some aspects of myotonic dystrophy (cataract formation, for example, is seen in chronic 20,25-diazacholesterol-treated animals), it is not the best model of myotonic dystrophy [705].

Before we close our discussion on azasterols, a note may be taken of the study of nucleo-aza analogues of cholestane which have been shown to possess hypocholesterolemic activity [706—708]. 3ξ-Ethyl-4-methyl-4-aza-5α-cholestane lowered plasma levels of cholesterol by 30% when administered subcutaneously to male rats at 20 $mg \cdot kg^{-1}$ [708]. This type of compound caused a build-up of desmosterol in the plasma and liver of mice and rats [706]. 4-Aza-5-cholesteno[3,4-b]-3′,4′,5′,6′-tetrahydropyrimidine (155) is also reported to lower plasma cholesterol levels in rats [642]. Metal chelate steroid analogues (165) [709] and (166) [710] have been shown to exhibit 58% and 65% *in vitro* hepatic cholesterogenesis inhibition, respectively.



(165) en = $H_2NCH_2CH_2NH_2$ (166) en = $H_2NCH_2CH_2NH_2$

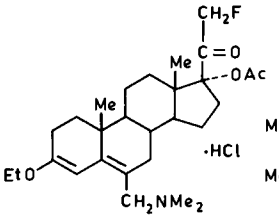
CENTRAL NERVOUS SYSTEM ACTIVITY OF SOME HETEROSTEROIDS

There are several examples known of hetero analogues in which properties like analgesic, anaesthetic, sedative, anti-convulsant and anti-depressant activities have been detected. There has not, however, come forth any derivative of clinical utility.

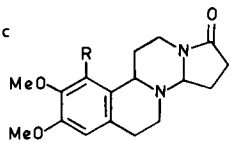
ANALGESIC ACTIVITY

The heterosteroid (SC-17599) (167) has been found to possess marked analgesic activity in several assays [711]. Nalorphine antagonizes respiratory depression induced by it but not the analgesic effect. Structurally SC-17599 has certain similarities with morphine. 6α-(Dimethylaminomethyl)-5-cholestane-3β,5α,6-triol is claimed to possess analgesic activity [695].

16β-Amino-17α,20β-dihydroxy and 16β-amino-17α-hydroxy-20-oxopregnenes have been reported to exhibit analgesic activity as measured by the writhing mouse assay [109,603]. 3α-Amino-5α-pregnane is also mentioned to have analgesic activity [712].



(167)



(168) R = H
(169) R = OMe

In preliminary testing the nucleo-azasteroids (168) and (169) showed some analgesic activity [713]. A related compound 2,3-dimethoxy-8,13-diaza-1,3,5(10)-gonatrien-12-one is also mentioned as an analgesic [714]. 4-Amino-2,3-dimethoxy-1,3,5(10)-estratrien-17-one was found to be inactive as an analgesic in the rat tail flick test [715].

OTHER ACTIONS ON CNS

Some 17-hydroxyimino steroids, for example, 4-androstene-3,17-dione 17-oxime and 4-androstene-3,11,17-trione 17-oxime (adrenosterone 17-oxime) produce a long-acting anaesthetic action in mice, when administered intraperitoneally at a dosage of 3 mg per mouse [670]. Reduction of 4-en-3-one grouping or another modification on ring A decreases the activity.

Certain amino steroids and other derivatives are claimed to possess anaesthetic properties: 6β-aminoandrostane-3β,5α,17β-triol [716], 6α-acetamidoprogesterone [717], 11β-dialkylaminopregnanes [718], 11β-aminoacetoxy steroids [719], amino acid esters of 21-hydroxypregnanedione [720].

During studies on steroids for their catatoxic activity, Selye [484,721] noted that certain steroids were highly potent anaesthetics when given

orally at the dose of 10 mg per 100 g rat. Among these were 17-hydroxy-3-oxo-4-estrene-17α-propionic acid γ-lactone (SC-8109), 17-hydroxy-3-oxo-4-androstene-17α-propionic acid γ-lactone (SC-5233), 11β,12β-oxido-5β-pregnane-3α, 20β-diol (SKF-5454), and 11α-methyl-11-hydroxy-5β-pregnane-3,20-dione dioxime.

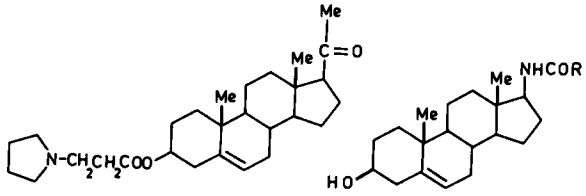
11β-Hydroxy-11α-methyl-5β-pregnane-3,20-dione dioxime has been shown to exhibit significantly greater CNS depressant activity than the parent dione [722]. It is not known if the effect is due to the intact molecule or to its breakdown products. 20β-(Dimethylaminoethoxy)-5-pregnen-3β-ol is a potent hypnotic (minimal-effective dose, 28 $mg \cdot kg^{-1}$ intravenously in mice) [723]. However, in doses smaller than those producing sleep, it exhibited marked side effects, *e.g.*, convulsions, dyspnoea. Ring A fused tetrazoles as 17β-hydroxy-3-aza-A-homo-4a-androsteno[3,4-d]tetrazole have been tested and found to possess weak central nervous system depressant activity in mice [724].

Funtumidine (3α-amino-5α-pregnan-20α-ol) was reported in 1960 to cause tranquillisation [725]. Certain synthetic 3α-aminopregnane derivatives [712,726] and 16-amino-17-hydroxy (or keto)-analogues [726] have been shown to possess tranquillising and anti-convulsant activities. Out of a series of 3α-hydroxy-2β-amino steroids tested, 3α-hydroxy-2β-morpholino-5α-pregnan-20-one was found to be the most potent in causing loss of righting reflex, the effect being possibly due to interneuronal blockage [727]. It exhibited anti-leptazol activity but no anti-electroshock or anti-tremorine response.

The hydrochlorides of 3β,5-dihydroxy-6β-morpholino-5α-androstan-17-one and its 3β-acetate, and 3β,5-dihydroxy-6β-morpholino-5α-pregnan-20-one and the 20β-ol induced sedation when administered intravenously in mice at 100 $mg \cdot kg^{-1}$ [728]. At the same dose level these compounds also showed anti-convulsant activity by protecting mice against electrically-induced seizures. In marked contrast, the 3β-acetate of 3β,5-dihydroxy-6β-morpholino-5α-pregnan-20-one was almost equipotent with leptazol as a convulsant [727,728]. 3α-Amino-4-pregnen-20-one, 17β-hydroxy-16β-piperidino-5α-androstan-3-one and 16β-piperidino-5α-androstane-3β,17β-diol are also convulsants [726].

Among different 3β-(aminoalkyl) esters of pregnenolone examined for anti-convulsant activity, the most active was SC-10024 (170) [729]. It was found to be devoid of hormonal effects. A brief clinical trial showed SC-10024 to exert beneficial effects in patients with epilepsy, but it did not get any further attention because of its low activity.

Some 4-azaestranes tested such as 17β-hydroxy-17α-methyl-4-azaestran-3-



(170) (171)

one showed anti-depressant activity in mice and shortened the duration of anaesthesia when they were administered 24 h before the anaesthetic [726]. When given only one hour before the anaesthetic, they had the opposite effect. They exerted their anti-depressant effect by augmenting the elimination of the anaesthetics both from the brain and from the whole carcass.

17β-Aminoacylamido derivatives (171) have been found to have anti-depressant activity in screening tests in mice [730,731]. The L-alaninamido, β-alaninamido and L-threoninamido derivatives [731] showed weak to fair activities comparable with the active L-serinamido and other earlier reported derivatives [730]. There seems to be no structural requirement for a natural amino acid residue in the side chain. Active compounds were obtained with either the L-seryl or the D-seryl residue in the side chain.

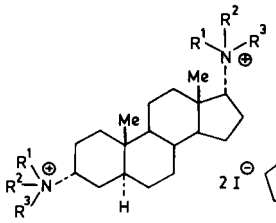
A study with the steroid alkaloid solasodine has shown it to decrease the temperature of normothermic rats [732]. In mice, the influence was even somewhat more intensive and longer-lasting than in rats. Hyperthermic state produced by pyrogens or with 2,4-dinitrophenol was normalised with 1 mmole·kg^{-1} solasodine in all animals. The observations imply a possible central site of action.

NEUROMUSCULAR BLOCKING AZASTEROIDS AND SOME GANGLIONIC BLOCKERS

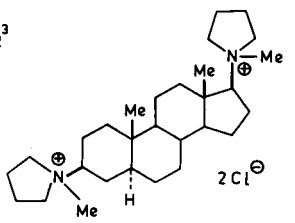
The literature on neuromuscular blocking azasteroids has been reviewed earlier [78,733,734]. The following treatment gives a brief description of the prominent features. At the end, mention is made of some ganglion blocking compounds.

MALOUETINE TO PANCURONIUM AND ALLIED ASPECTS

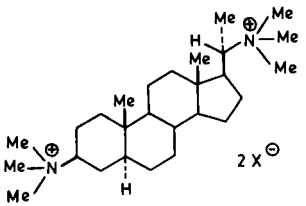
The steroid nucleus as a supporting moiety for two cationic heads has been considered to be a proposition worthy of interest. The nucleus is



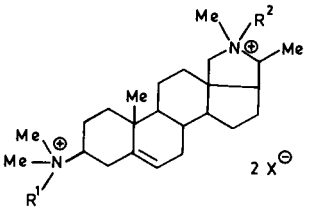
(173)



(174)



(172)



(175)

relatively rigid and as such the spatial relationship between two onium centres would be more or less fixed and there will be a limited flexibility through conformational variations. That such compounds would possess appropriate hydrophilic to lipophilic ratios was apparent from the discovery of neuromuscular blocking activity comparable with that of (+)-tubocurarine in the steroidal alkaloid malouetine (172) [735,736] and its configurational isomers at C-3 and C-20 [737-739]. In these, a degree of variation in interonium distance, 11 to 12.5 Å, can be visualised due to free rotation of the side chain, and as such a study of the bisonium azasteroids having both quaternary ammonium groups directly attached to the nucleus was considered worthwhile.

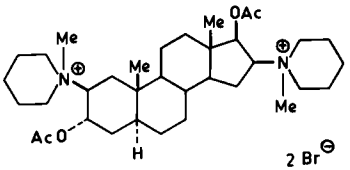
A series of 3*α*,17*α*-bis(quaternary ammonium)-5*α*-androstane (173) [740], in which the interonium distance (9.2—10.6 Å) was near the favourable range, and steric hindrance to post-junctional binding by *β*-face angular methyl groups on C-10 and C-13 was excluded, showed activity, though less than that of (+)-tubocurarine. On the basis of studies on dipyrandium chloride (174) [741,742] and all the eight of its isomers [742,743], tests being made *in vivo* on the cat or monkey sciatic nerve tibialis muscle preparation, it was seen that 3*β* isomers were in general more potent than the corresponding 3*α* compounds, and there was no general relationship

between potency and interonium distances. On this basis, Bamford, Biggs, Davis and Parnell [742] tended to support the adumbration theory of Loewe and Harvey [744], who postulated a one-point attachment theory, where the bulk of the molecule, in this case presumably the steroid nucleus, shields the receptor, rather than the suggestion of Cavallito and Gray [745] and Waser [746] that a two-point receptor complex could be formed. The relatively flat steroid nucleus in the 5*α*-series may be a more effective shield than the more folded nucleus in the 5*β*-series, and therefore the 5*α*-series should be more potent than the corresponding members of the 5*β*-series, and that indeed was seen to be the case. By testing several monoquaternary androstane derivatives, Bamford, Biggs, Davis and Parnell [747] further supported the one-point-attachment theory, and again considered a quaternary centre linked to position 3 to be more important than one attached to position 17 in determining potency in the series.

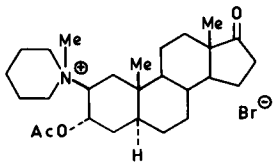
The activity of the steroidal quaternary compounds examined has been of non-depolarising type. It was also so with the quaternary salts (175) derived from the alkaloid conessine [748]. In the cat most of these compounds possessed short-acting muscle relaxant properties; block duration was shorter, and cumulation was less than with gallamine or (+)-tubocurarine. The bisquaternary compounds reported possess the interonium distance of 10.1 Å. Seven of the eight 3-monoquaternary compounds tested were also potent neuromuscular blocking agents; this observation may not be taken as a convincing evidence for one-point-attachment since the second nitrogen could become protonated in the system and thus provide the second cationic head.

Though there is lively discussion as to the mode of post-synaptic attachment, interest in the steroid quaternary compounds continues. Certain drugs thus evolved have shown promise on clinical testing. These include dipyrandium chloride (174) [749], *N,N*-dimethylconessine (175; $R^1 = R^2 = Me$) [750] and pancuronium bromide (176) [751—754]. Pancuronium bromide (Pavulon) is marketed and advocated for use in clinical situations where a non-depolarising muscle relaxant of medium duration of action is required, due to its high potency with minimal side effects. A brief description may be given about the design of pancuronium bromide [755] and some ancillary aspects.

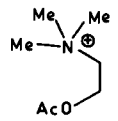
A programme on the synthesis and pharmacological study of 2*β*-amino-3*α*-hydroxy-5*α*-androstanes and derivatives [756] and the corresponding 3*α*-amino-2*β*-hydroxy isomers, led to the observation [757] that the corresponding monoquaternary salts possessed neuromuscular blocking activity; the most potent of the series, 3*α*-acetoxy-2*β*-piperidino-5*α*-androstan-



(176)



(177)



(178)

17-one methobromide (177), has one-sixteenth the potency of (+)-tubocurarine. The 2β-piperidino and 3α-acetoxy group are both considered to be pseudoequatorial due to the twisted boat conformation of ring A [756]. In this preferred conformation which may be rigid due to steric compression [758], one may consider it to have ring A substituents in specific molecular conformation akin to the neurotransmitter acetylcholine (178), and thus (177) may be expected to occupy the transmitter's site of action and neuromuscular transmission. As the monoquaternary analogue (177) had only a low activity, it was thought that a bisquaternary azasteroid may be potent and pancuronium bromide (176) was ultimately synthesized [755] and tested [759]. Here also the 16- and 17-substituents are pseudoequatorial.

Structure-activity studies [755] on pancuronium bromide and other steroidal neuromuscular blocking agents containing acetylcholine fragments indicated that for high potency it is probably essential to have two nitrogen atoms in the molecule and at least one of these nitrogen atoms should be quaternized. X-ray crystallographic studies [758] with pancuronium bromide reveal the actual interonium distance in the solid state to be 11.08 Å as against 10.6 Å calculated from Dreiding models.

A relative of pancuronium bromide, dacuronium bromide (2β,16β-dipiperidino-5α-androstane-3α, 17β-diol 3α-acetate dimethobromide) [755] on clinical studies [760,761] showed that it possessed a rapid onset and shorter duration of action than that of pancuronium bromide but it lacked sufficient potency to be a clinically-useful drug.

Dacuronium differs from pancuronium (176) in having unacylated free hydroxyl at position 17. Another analogue Org 6368 (2β,16β-dipiperidino-5α-androstan-3α-ol acetate dimethobromide) has no substituent at position 17. It is less active than pancuronium but is rapid in onset and has shorter duration of action as that of pancuronium [762]. The short duration of action of Org 6368 may be due to its early hepatic uptake [763].

Compounds related to pancuronium (176) but having 2β, 16β-dipiperizino functions in place of piperidino groups are also claimed to be active as neuromuscular blockers [764].

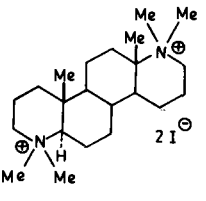
Pancuronium is a drug of interest and work on its pharmacology and therapeutic use continues. There is experimental evidence to show strong binding of pancuronium to gamma globulin and of moderate binding to albumin [765]. Renal elimination appears to be the major excretory pathway in mice [766], rats [767] and humans [768—770]. On average, half of the injected dose is recovered in the urine; 80% of this proportion is unchanged pancuronium and 20% deacetylated metabolites [770]. The biliary excretion 24 h after the administration of pancuronium accounts for 5 to 10% of the injected dose. Renal and biliary elimination as well as the rate of metabolic deacetylation exhibit wide individual variations. Metabolic degradation and biliary elimination do not influence the duration of neuromuscular block. The renal failure can affect the duration of action of pancuronium bromide [771].

CHANDONIUM AND RELATED STUDIES

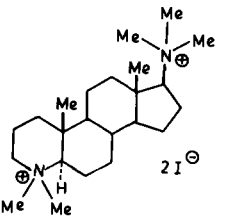
The search for a short-acting non-depolarising neuromuscular blocking agent continues, and the azasteroidal area is worthy of further investigations in this quest.

Singh's group at the Panjab University Department of Pharmaceutical Sciences 'initiated a programme of synthesis of bis-onium steroids as potential neuromuscular blocking agents, with one or both of the cationic systems present as part of the steroid ring skeleton at different interonium distances' [772] and first designed 4,17a-dimethyl-4,17a-diaza-D-homo-5α-androstane dimethiodide (HS-342) (179) [772]. In the anaesthetized cat, HS-342 exhibited non-depolarising neuromuscular blocking activity approximately equal to that of (+)-tubocurarine, but in contrast exhibited a short duration of action (one-third of that of (+)-tubocurarine) and rapid onset of action [773,774]. HS-342 also possessed a ganglion-blocking activity. This combination of blocking actions is probably related to interonium distance, 8 Å (derived from Dreiding models), which falls between the optima for these two activities. HS-467 (180) [775] which has the interonium distance (9 Å), somewhat extended as compared with HS-342, was approximately equipotent to (+)-tubocurarine but exhibited extremely weak ganglion blocking activity [776] and duration of action was again short and onset rapid.

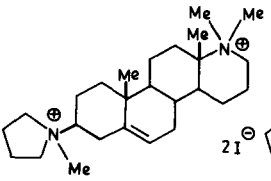
In the series, a compound of particular interest was chandonium iodide (HS-310) (181) [775]. It possesses a powerful non-depolarising neuromuscular blocking activity of short duration and rapid onset, being only slightly less active than pancuronium [776—778]. It has little or no ganglion blocking



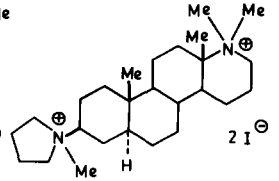
(179)



(180)



(181)



(182)

activity. The interonium distance in chandonium iodide is 10.2 Å (Dreiding models). X-ray diffraction studies have been carried out with 17a-methyl-3β-pyrrolidino-17a-aza-D-homo-5-androstene (HS-309) [779], the tertiary amine corresponding to chandonium iodide (181). The N—N separation in HS-309 is 10.14 Å; the rings A, C and D in chair conformation and B in a half-chair conformation. The results obtained so far suggest that chandonium may have possible clinical applications as a short-acting muscle relaxant.

Using chandonium as a prototype, further chemical modifications have been made. The saturated congener dihydrochandonium iodide (HS-692) (182) and the analogues possessing bulkier cationic heads have been synthesized [780]. The saturation of 5,6-double bond in chandonium (181) and increase in the onium bulk in (181) or (182) diminishes the potency [781]. Still, dihydrochandonium iodide (182) is a compound of interest, having high potency (half of chandonium), short-lasting neuromuscular block, no ganglion block and the least vagolytic action, in the anaesthetized cat.

SOME GANGLION BLOCKERS

In the bisquaternary ammonium compounds, it is generally accepted that the optimal interonium distance for neuromuscular blocking activity is in the region of 10—12 Å. Ganglion blocking activity does not appear to

be so dependent upon interonium distance, but as the interonium distance falls below 10 Å, so the neuromuscular blocking activity diminishes, with the result that the ganglion blocking activity becomes more predominent. The bisquaternary ammonium salts derived from 3*α*,12*α*-diamino-5*β*-cholane and 3*α*,12*α*-diamino-24-nor-5*β*-cholane and possessing an interonium distance of ca 5.8 Å are also active ganglion blockers [782].

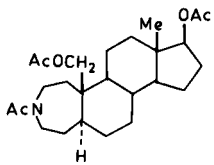
Certain monoquaternary azasteroids, for example, 17a-methyl-17a-aza-D-homo-5-androsten-3*β*-ol methiodide (HS-308) and 4-methyl-4-aza-5*α*-androstan-17*β*-ol methiodide (HS-419) are equipotent with hexamethonium as ganglion blockers [776].

HETEROSTEROIDS WITH LOCAL ANAESTHETIC ACTIVITY

There are only a few reports of heterosteroids possessing local anaesthetic activity.

Local anaesthetic properties have been reported for steroidal alkaloids [783,784]. With conessine, isoconessine and neoconessine, local anaesthesia was found after intracutaneous injection in guinea-pigs, but the irritation after injection and other side effects precluded any further interest. Local anaesthetic effect has also been claimed for funtumidine (3*α*-amino-5*α*-pregnan-20*α*-ol) and funtumine (3*α*-amino-5*α*-pregnan-20-one) [784].

A study of 3*α*-amino-2*β*-hydroxy and 2*β*-amino-3*α*-hydroxy-5*α*-androstanes [756] has revealed compounds with potent local anaesthetic properties [785]. A particular mention may be made of the analogue Org NA13 (152) [635]. It was shown to possess more potent local anaesthetic properties than lignocaine in guinea-pig after intracutaneous and surface applications. The conduction blockade as demonstrated in the rat sciatic-nerve *in vivo* was similar for both NA13 and lignocaine. The onset of the blockade was less rapid after NA13. In contrast to lignocaine, other local anaesthetics and *β*-adrenoceptor blocking drugs, Org NA13 did not show any activity against the arrythmias induced by ouabain in dogs. Local anaesthetic activity is claimed in some *N*-acetyl-3-aza-A-homoandrostanes [786, 787]. The analogue (183) is said to be useful as a local anaesthetic.



(183)

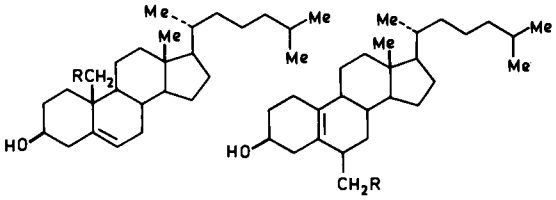
SCINTISCANNING STEROIDAL DIAGNOSTIC AGENTS

Radioiodinated steroids have been of interest as an approach to prepare analogues for photoscanning the adrenal gland and associated tumours. As it had been known that uptake of radiolabelled progesterone by the adrenal gland far exceeded that of any other tissue [788,789], 21-[^{125}I] iodoprogesterone and 21-[^{125}I] iodopregnenolone acetate were synthesised as possible adrenal scanning agents [790]. However, these rapidly underwent *in vivo* deiodination.

Adrenals are rich in cholesterol and labelled cholesterol has been found to selectively concentrate in the adrenal tissue [791—793]. ^{131}I-labelled Cholesterol was prepared by treating cholesterol with ^{131}I-labelled sodium iodide and chloramine-T [794]. The product, possibly a 5,6-double addition product, was not characterized. Radioiodinated stigmasterol was also prepared. But *in vivo* deiodination problem remained as seen in distribution studies in mice.

Considering that C-19 position of cholesterol may better withstand *in vivo* dehydrohalogenation, prompted the synthesis of 19-[^{125}I] iodocholesterol [795]. It was found to be much less prone to rapid *in vivo* deiodination. The concentration of radioactivity in adrenal cortex of dogs at 48 h was found to greatly exceed that found in other organs. ^{131}I-labelled 19-iodocholesterol (184) has been widely recognized clinically as a diagnostic agent for scintiscanning of the adrenal gland and associated tumours [796—808]. The value of 19-[^{131}I] iodocholesterol has been shown in the assessment of patients suspected of having Cushing's syndrome [796,802, 803].

It has been pointed out that 19-[^{131}I] iodocholesterol [795] always contain considerable amount of an impurity which was isolated and identified as ^{131}I-labelled 6β-iodomethyl-19-nor-5(10)-cholesten-3β-ol (185) [809—811]. Homoallylic rearrangement of (184) gives (185) [809,810]. Procedures for the preparation of ^{131}I-labelled (184) [812] and (185) [813] of high purity have been described.



(184) R = I (185) R = I

(187) R = Me-Se- (186) R = Br

It has been found that ^{131}I-labelled (185) accumulates to a greater extent (5—10 fold) in the adrenal gland than labelled (184) [814—816]. It has been suggested that ^{131}I-labelled (185) is more suitable as an adrenal scanning agent than (184), and the clinical usefulness of the former for adrenal imaging has been reported [817—819]. The bromo (186) and chloro analogues have been prepared [820]. The ^{82}Br-labelled (186) has been also synthesized and tissue distribution in rats studied, but it has not been found superior to the corresponding ^{131}I-analogue (185) [821].

^{75}Se-labelled 19-selenocholesterol (187) has shown high adrenal concentrations and good adrenal images in tissue distribution studies in rats, rabbits and dogs [822]. In the dog, higher concentrations were obtained in adrenal medulla than in the cortex. This new adrenal scanning agent is being evaluated in humans.

Lastly, a mention may be made of the studies with ^{75}Se-labelled 2-selena-A-nor-5α-androstan-17β-ol [823]. This agent complexes with the specific receptors of 5α-dihydrotestosterone (5α-DHT) in the prostatic cytosol and is retained by the nuclei. Its uptake pattern and localisation are similar to that of 5α-DHT. It appears that the ^{75}Se analogue of high specific activity could demonstrate the feasibility of using appropriately radiolabelled androgens as prostatic imaging agents.

AZASTEROIDS AND ANTIMICROBIAL ACTIVITY

A good number of azasteroids have shown antibacterial and antifungal activities. In some, antiprotozoal and antiviral activities have been found. The discussion which follows is based on structural classifications.

ANTIMICROBIAL AMINOSTEROIDS AND SOME SIMPLE HETERO DERIVATIVES

Many simple derivatives of steroids have been tested. Some derivatives of sulphanilamide with cholic acid were made [824]. N^2-*p*-Aminobenzenesulphonyl cholic acid hydrazide had considerable bacteriostatic activity against *Streptococcus haemolyticus* and *St. pneumoniae*.

Testosterone isonicotinylhydrazone [825] and thiosemicarbazones of testosterone and progesterone [826] were active as antibacterials. Certain 17-dimethylaminoethoxyimino steroids have been shown to be active against Gram-positive bacteria, *Mycobacterium tuberculosis*, or fungi [670]. The methoximino derivative of 3β-hydroxy-5α-androstan-17-one is reported to be

as potent as griseofulvin and its antifungal spectrum is broader. Some steroid glyoxals (21-al-20-ones) and their Schiff's bases and *N,N*-diacetals proved to be active against influenza A-PR8 virus in chick embroys [827].

Various cholestane, cholane, pregnane and androstane derivatives bearing extranuclear basic nitrogen at different positions have been studied. 7*α*- and 7*β*-Aminocholesterol and 7*α*-aminocholestanol showed appreciable antibacterial activity against Gram-positive organisms [828]. However, 3,7-diamino-12-oxocholanic acid and 7-amino-3,12-dihydroxycholanic acid which have free 24-carboxylic group were inactive [829]. But the basic 23-aminonorcholane [829,830] and 24-aminocholane derivatives [829] were active. The most potent compound was 23-guanidino-3,7,12-trihydroxynorcholane [829]. The basic 3-amino-7*α*,12*α*-dihydroxycholanic esters were also active, the *n*-hexyl ester being the most potent [831]. A study of 3-, 6- and 7-amino substituted cholestanes has shown that the diamino derivatives have consistently higher antibacterial potency, as compared with monoaminosteroids, against Gram-positive bacteria [832]; moreover, the group of diamino compounds also showed considerable bacteriostatic activity against the Gram-negative organisms tested.

Several amines and their quaternary derivatives prepared from 3*β*-acetoxybisnor-5-cholenic acid have been found to possess the ability to inhibit the growth of *Candida albicans* [833]. Some 3*β*-dialkylamino derivatives of cholestane and cholane, and 23-dimethylamino norcholane-3*α*,7*α*,12*α*-triol when tested did not show sufficient amoebicidal activity [834].

3*β*-Acetoxy-21-dimethylamino-5,16-pregnadien-20-one hydrochloride and 3*β*-acetoxy-21-dimethylamino-5-pregnen-20-one methiodide inhibited the growth of *Bacillus subtilis* [835]. The former compound and its 21-morpholino analogue showed significant fungistatic activity against *Aspergillus niger*. 21-Dimethylaminomethyl-5*α*-pregnan-3*β*,20*β*-diol dipropionate hydrochloride is reported to be a potent bacteriostatic agent [836]. Some 20-dimethylamino-5*α*-pregnane derivatives have been claimed to inhibit growth of *Escherichia coli* and *M. tuberculosis* [837].

Several 16*β*-amino-17*α*-hydroxypregnane derivatives have been prepared and tested for antimicrobial activity [109,638]. For example, 16*β*-ethylamino-5-pregnene-3*β*,17*α*, 20*β*-triol and its 16*β*-(4-methylpiperazino) analogue showed antibacterial activity against *Diplococcus pneumoniae*, and exhibited complete immobilization (death) of the protozoan *Tetrahymena geleii* [638]. 17*α*-Hydroxy-16*β*-methylamino-4-pregnene-3,20-dione hydrochloride was active as an antiprotozoal, antifungal and anti-algal [109].

17*β*-Amino-3,5-androstadiene and 17*β*-amino-5-androstene were active in

testing against *Trichophyton asteroides* and *Penicillium citrinum* [110]. *N*-Substitution lowered the activity. Even 3-hydroxyl function was not conducive for activity, since 17β-amino-5-androsten-3β-ol and its *N*-substituted analogues were inactive against the two organisms. Certain 17-amino-1, 3,5(10)-estratrienes have been studied for their antimicrobial activities [838].

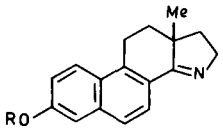
A mention may be made of ring A seco aminosteroids. The compounds 3-amino-3,5-seco-A-norandrostan-17β-ol, 2-amino-2,5-seco-A-dinorandrostan-17β-ol and 17β-hydroxy-2,5-seco-A-dinorandrostan-2-yl guanadinium acetate have shown moderate activity against *Mycobacterium tuberculosis* [839].

NUCLEO- AND OTHER CYCLIC AZASTEROIDS POSSESSING ANTIMICROBIAL ACTIVITY

First, a reference may be made to a new group of naturally occurring mould metabolites isolated from *Geotrichum flavo-brunneum* [840]. This complex of naturally related compounds has strong antifungal properties [39,841], and marginally active against bacteria. The major factor has been identified as 15-aza-D-homo-5α-ergosta-8,14,24 (28)-trien-3β-ol (A25822B) (8) [842]. It was observed that when combined with the polyene antibiotic pimaricin, the azasterol (8) antagonizes the antifungal properties of the polyene [841]. The azasterol (8) has been shown to inhibit sterol transmethylation in the yeast *Saccharomyces cerevisiae* [843]. Different derivatives have been prepared from (8) by changes with the 24-methylene function or saturation of 14,15-double bond and claimed to possess antifungal activity [844—846].

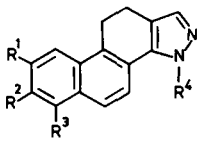
Some steroid related synthetic hetero analogues possessing nitrogen in ring D have been prepared and tested for antimicrobial activity. The 15-azasteroids hydroxyimine (188) and methoxyimine (189) [847] inhibit the growth of *B. subtilis* and *E. coli* at concentrations as low as 10^{-5}M [848]. The site of action is at the cell periphery. Both show synergistic antimicrobial action with chloramphenicol, actinomycin D, polymyxin, and circulin [849]. Combining the methoxyimine with polymyxin or circulin at subinhibitory concentrations produced greatly enhanced antimicrobial activity against *Pseudomonas fluorescens* [848]. Similar action was observed against *B. subtilis* when the azasteroid was combined with vancomycin or chloramphenicol. This suggests formation of a molecular complex between the azasteroid and antibiotic which is responsible for the enhanced biological activity.

In cell culture experiments (188) and (189) inhibited the growth of KB



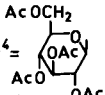
(188) R = H

(189) R = Me

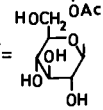


(190) $R^1 = R^3 = R^4 = H$; $R^2 = OH$

(191) $R^1 = R^3 = H$; $R^2 = OMe$; R^4 =



(192) $R^1 = R^3 = H$; $R^2 = OMe$; R^4 =



(193) $R^1 = R^2 = OH$; $R^3 = R^4 = H$

(194) $R^1 = R^2 = OMe$; $R^3 = R^4 = H$

(195) $R^1 = R^4 = H$; $R^2 = R^3 = OMe$

(196) $R^1 = R^2 = R^3 = OMe$; $R^4 = H$

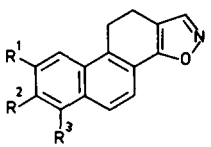
and L-M cells [850]. The former is more inhibitory, it increases the lysosomal membrane permeability and reduces transport into the cells of macromolecular species, protein, DNA and RNA.

Studies with (188) on *in vitro* mouse fibroblast cell permeability and on respiration of isolated rat liver mitochondria have been carried out [851]. It is suggested that the azasteroid may be acting directly on the electron transport system and/or acting indirectly through membrane perturbations which disrupts the electron transport process.

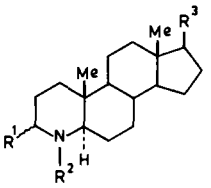
The analogues with ring D pyrazole and isoxazole systems have also been studied. The 15,16-diaza analogue (190) [852] specifically interacted with actinomycin D to form a molecular complex [853,854]. The combination has shown certain inhibition of selected micro-organisms and tissue culture cell lines [855]. The *N*-glucoside derivative (191) was active against *B. subtilis* and KB cells [856,857]. In contrast, (192) with the sugar in the free form (deacetylated) showed no activity [857]. The diol (193) was also inactive; however the methoxy analogues (194), (195) and (196) did inhibit *B. subtilis* but were ineffective against *P. fluorescens* [857,858]. The derivative corresponding to (193) but having *p*-fluorophenyl group attached to N of the pyrazole produced an inhibition of growth of both *B. subtilis* and KB cells [857]. The dimethylaminoethyl and *n*-hexyl ethers of (190) were effective against *B. subtilis*, but surprisingly the methyl ether of (190) was not very inhibitory with this organism but had a modest influence on the growth of *P. fluorescens* [859].

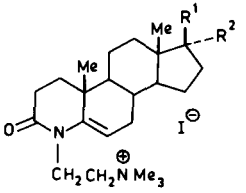
The isoxazoles (197) [857] and (198) [859] were active against *B. subtilis*, whereas the analogue (199) [858] was inactive.

Several aza analogues prepared by Doorenbos group by partial synthesis have been examined for their antimicrobial activity. Out of a variety of nitrogen-containing steroids tested, 4-aza analogues 3ξ,4-dimethyl-4-aza-5α-cholestane (200), its methiodide and 3ξ-benzyl-4-methyl-4-aza-5α-cholestane methiodide were found to have significant antimicrobial activity [860]. Sensitivity to the compounds was greatest in the Gram-positive bacteria, followed by the yeasts and moulds; the Gram-negative bacteria were not inhibited. These inhibited *St. pyogenes* and were completely inactivated by 0.1% lecithin [861]. 4-Dimethylaminoethyl-4-aza-5-cholesten-3-one methiodide (201) and 4-ethyl-4-aza-5α-cholestane (202) were very active as antibacterial against *B. subtilis* and *Sarcina lutea* [862]. The corresponding pregnane (203) and androstane (204) derivatives were inactive. Compound (201) had no effect on the growth of *E. coli.*



(197) $R^1=R^2=OMe$; $R^3=H$
(198) $R^1=R^3=H$; $R^2=-OC_6H_{13}$
(199) $R^1=R^2=R^3=-OMe$





(200) $R^1=R^2=Me$; $R^3=C_8H_{17}$
(202) $R^1=H$; $R^2=Et$; $R^3=C_8H_{17}$

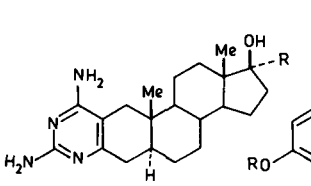
(201) $R^1=C_8H_{17}$; $R^2=H$
(203) $R^1=Ac$; $R^2=H$
(204) $R^1=OH$; $R^2=Me$

4,6β-Dimethyl-4-aza-5α-cholestane had decreased activity as compared with (200) and 4-methyl-4-aza-5α-cholestane [863]. It has been noted that polar functional group in the vicinity of the 17-position greatly decreased the antimicrobial potency [860,864]; 17β-amino grouping almost completely abolished the activity [865]. However, 4-(2-guanidinoethyl)-17α-methyl-4-aza-5α-androstan-17β-ol was active against *Saccharomyces cerevisiae* [866]. Potent antimicrobial 4-aza- compounds with ester and ether groups in the 17- and 20-positions have been obtained [867,868]. Certain 4-aza-5-cholestenes possessing partly or fully saturated pyrimidino and imidazolo rings

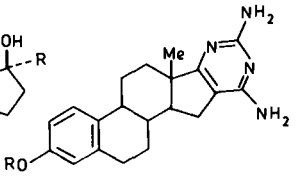
fused to 3,4-position were active against Gram-positive bacteria and fungi [642].

The location of nitrogen from position 4 may be moved to positions 3 or 2 without loss of activity. 2-Methyl-2-aza-5α-cholestane and 3-methyl-3-aza-5α-cholestane are reported to possess antimicrobial properties similar to those of 4-methyl-4-aza-5α-cholestane [869]. In the 17a-aza series, none of the *N*-alkyl and *N*-alkylaminoalkyl derivatives of 17a-aza-D-homo-5-androsten-3β-ol exhibited significant antibacterial or antifungal activity [870]. A series of 4-aza-3,5-cyclocholestane [871] and 6-aza-5,7-cyclocholestanol [872] derivatives are claimed to possess antifungal properties. 2-Azaestratrienes obtained by total synthesis have been tested as antiviral agents. The 3-methyl ether series exhibited anti-influenza activity *in vitro* [49]. Compounds of 1,3-dimethoxy series were devoid of anti-viral properties.

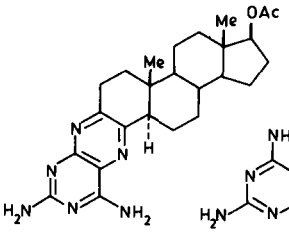
There have been prepared a number of aza analogues having heterocyclic rings fused to the steroid nucleus. The steroidal[3,2-d]- (205,206) and [17,16-d]-2′,6′-diaminopyrimidines (207,208) were found to be active against several Gram-positive organisms, and particularly against *S. aureus* strains [317]. However, neither (205) nor (207) protected mice challenged intraperitoneally with a penicillin-resistant strain of *S. aureus* CHP. Some steroidal[2,3-e]-1′,2′,3′,4′-tetrahydropyrazines have been examined as antimicrobials, but found to be inactive [298].



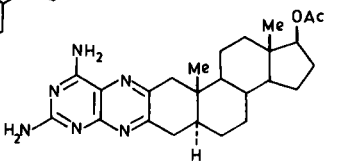
(205) R = H
(206) R = Me



(207) R = H
(208) R = Me



(209)



(210)

Certain pteridinosteroids have been synthesized with the hope that they may combine the antifolic activity of 2,4-diaminopteridines with lipid solubility and with the favourable cellular transport properties of steroid molecules. The compounds (209) [373] and (210) [374] are moderately active antifolinic agents as tested using *Lactobacillus leichmannii* system, the latter being relatively more active.

The bridged heterocycles 3α,5-iminomethano- and 3β,5-iminomethano-5β-cholestane were found to be inactive against Gram-negative bacteria but active against Gram-positive bacteria and *Trichomonas vaginalis* [873].

Lastly, a mention may be made of some naturally-occurring azasteroids which possess nitrogen as part of a cyclic structure other than nucleoplacement. Conessine, an alkaloid of *Holarrhena antidysenterica* is known for its anti-amoebic activity [874]. Tomatine, a glycoalkaloid has been known to exhibit antifungal and anti-bacterial activity [875,876]. Solacasine, an alkaloid has been reported to be the main anti-bacterial constituent of *Solanum pseudocapsicum* [877].

ANTI-NEOPLASTIC HETEROSTEROIDS

Certain steroidal nitrogen mustards, nucleohetero derivatives and analogues with fused heterocyclic systems have been found to be of particular interest and have been tested clinically. The survey which follows does not include naturally-occurring heterosteroidal systems which have shown anti-neoplastic activity.

STEROIDAL NITROGEN MUSTARDS AND AZIRIDINES

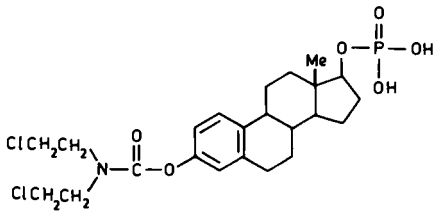
It has been hoped that the passage of the alkylating agent across cell membranes might be facilitated by linking it to a more lipophilic moiety, like a steroid. The use of hormonally-active steroids for this purpose might direct the mustard to specific target tissues. Further, it has also been surmised that in the case of the sex hormones and glucocorticoid derivatives, may be two cytotoxic actions are achieved, one resulting from hormone-receptor interaction and the second due to a nitrogen mustard effect.

In 1952 the first nitrogen mustard derivatives of cholesterol were prepared [878,879]. The analogues having nitrogen mustard function attached to cholestanes, androstanes and estratrienes were synthesized [880—885] but these showed no promise.

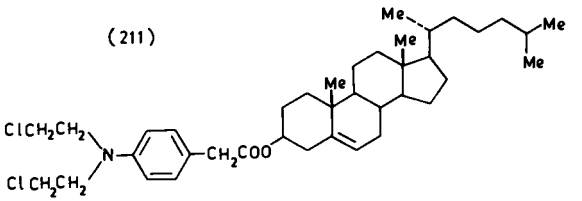
Attempts were then made to obtain latent anti-neoplastic compounds by

attaching the *N,N*-bis(2-chloroethyl)-carbamoyl moiety to the steroid nucleus of cholesterol [886], estrone [887], estradiol and 7*α*-ethinylestradiol [888], testosterone [889], androsterone and epiandrosterone [888] and hydrocortisone [890]. Testosterone 17*β*-[*N,N*-bis(2-chloroethyl)]carbamate exhibited 75% inhibition of mammary adenocarcinoma MI [891] and 17*α*-ethynylestradiol3-[*N,N*-bis(2-chloroethyl)]carbamate caused 60% inhibition of Walker 256 carcinosarcoma in rats [888]. It appears that the urethane type nitrogen mustard derivatives with anti-tumour activity may retain hormonal properties. 21-[*N,N*-Bis(2-chloroethyl)]carbamate derivatives of triamcinolone acetonide and hydrocortisone are active as inhibitors of the growth of cultured mouse fibroblasts and are able to compete for the specific binding of radiolabelled triamcinolone acetonide to the L929 cell receptor [892].

Estramustine phosphate (Estracyt) (211) has been used in the treatment of carcinoma of prostate [893—895]. However, further clinical trials are warranted [896]. The mechanism of action is obscure. *In vivo* studies of estramustine phosphate have shown that it lowers prostatic 5*α*-reductase activity, causes decreased prostatic weight and secretions, and when administered for 2 days stimulates in the uptake of labelled 5*α*-dihydrotestosterone into the prostate [897,898]. The carbamate ester linkage can be hydrolysed *in vivo* to form free estradiol; however, the site of this hydrolysis is not known [899]. There is little evidence for a direct effect of estracyt separate from oestrogen effects produced by hydrolysis occurring at a site other than the target organ [900]. Estriol 3-[*N,N*-(2-chloroethyl)]carbamate 17-phosphate disodium salt has also been found to have anti-prostatic effect in dogs and rats [901].



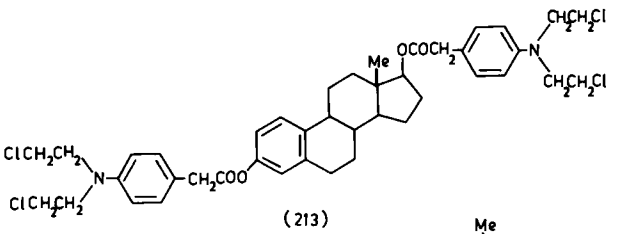
(211)



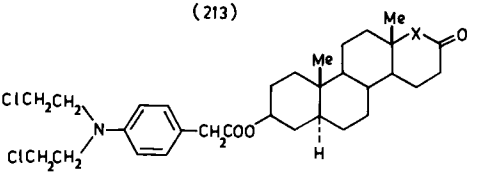
(212)

Russian workers [902—905] described the preparation of 5-cholesten-3β-yl *p*-[*N*,*N*-bis(chloroethyl)amino]phenylacetate (phenesterin) (212) and made studies of its activity against a variety of solid tumour systems including Sarcoma 45, Walker carcinosarcoma, and alveolar liver carcinoma RS-1 [902,903] and also in brain tumours [904]. At levels of 100—200 mg·kg^{-1} in rats and mice, and subcutaneous administration in olive oil, there was remarkable inhibition of the tumour systems; the compound was inactive against Ehrlich and Sarcoma 180 mice tumours. Phenesterin was found to be relatively nontoxic with an LD_{50} value for rats (single injection) of 2.0 g·kg^{-1} [902]. The Russian workers stressed the importance of ester function and claimed that the action of phenesterin was different from that of the parent free acid [903]. Phenesterin has recently been tested clinically [906,907].

Extensive studies were made on a series of steroid esters of *p*-[*N*,*N*-bis (2-chloroethyl)amino]phenylacetic acid (BCAPAA) [908,909], steroidal sulphides of *p*-[*N*,*N*-bis-(2-chloroethyl)amino]thiophenyl and a variety of steroidal ethylenimine derivatives [908]. Antitumour activity was found in those instances in which the steroid and potential oncolytic agent was connected by a more easily cleaved ester or heterocyclic ether linkage. The steroidal BCAPAA esters were of particular interest showing excellent inhibition of a DMBA-induced and transplantable mammary adenocarcinoma, and marked increase in survival when tested on a variety of rat leukemias. The nature of steroid, route of administration and vehicle have definite effects on activity. The estradiol diester (213) was selected for clinical studies [910].



(213)

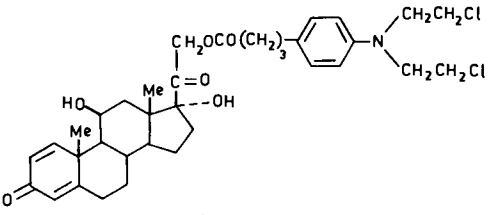


(214) X = -NH-

(215) X = -O-

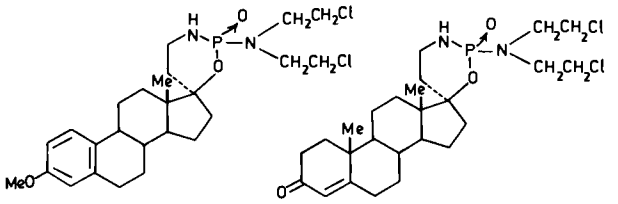
Recently there has been prepared BCAPAA ester (ASE) (214) [911], which has been shown to be active against L1210 and P388 leukemias in mice [912]. The oxa isostere (215) is less effective [913] than (214) [914] in tumour T8, melanoma B16 and Th-B angiosarcoma.

p-[*N*,*N*-Bis(2-chloroethyl)amino]phenylbutyric acid (BCAPBA, chlorambucil) esters of different steroids have also been synthesized. The 17-ester of testosterone was prepared but no test data have been reported [915]. The chlorambucil esters of cholesterol and estrone exhibited excellent inhibition of mammary tumour in rats — the BCAPBA ester appeared to be more active than its BCAPAA derivative (phenesterin) [909].



(216)

Prednimustine (Leo1031) (216), chlorambucil 21-ester of prednisolone, has been shown to be active against the L1210 murine leukemia model [916] and has been tried in patients with chronic lymphocytic leukemia [917] and lymphocytic lymphoma [918]. It has been demonstrated that it may also be useful in the treatment of acute leukemia [919]. It has been shown that prednimustine is more effective than chlorambucil against a sensitive line of the Yoshida ascites sarcoma but ineffective against Ehrlich ascites tumour which is intrinsically resistant to alkylating agents [920].



(217) (218)

The other kind of steroidal mustards prepared are in which cyclophosphamide moiety has been attached to the steroid nucleus [921,922]. The potential anti-neoplastic derivatives (217) [921,922] and (218) [922] have been prepared but no biological data have been reported.

16-Substituted aziridinomethyl and aziridino derivatives have been studied [923]. 16ξ-Aziridinomethyl-17-oxo-5-androsten-3β-yl acetate, 16ξ-aziridino-4-pregnene-3,20-dione and 16ξ-aziridino-3β-hydroxy-5-pregnen-20-one in C-3H mice implanted with mammary tumours have given inhibition of growth of about 50% (29—84%) in multiple assays when administered at a dose of 0.5 mg/mouse/day for 14 days.

OTHER HETEROSTEROIDAL SYSTEMS

A prominent anti-cancer nucleo-heterosteroid of interest is testolactone (101) which is hormonally inactive. Objective regression of metastatic breast cancer with (101), has been reported ranging from 12.5 to 30% [924—928]. It is effective for the palliative primary treatment of women with advanced breast cancer. No significant metabolic changes were detected in a study on three patients [929], but a fall in urinary calcium calculated to result from an increase in tubular reabsorption has been reported [927]. The predominent metabolite of testolactone is 3α-hydroxy-17a-oxa-D-homo-5β-androst-1-en-17-one [930,931].

Testololactone (1,2-dihydrotestolactone) is not effective as an anti-cancer agent [932]. 17a-Oxa-D-homo-1,4,6-androstatriene-3,17-dione is claimed to be useful in mammillary carcinoma [933].

2-Azaestradiol 3-methyl ether 17-acetate was active in the L1210 assay (mouse leukemia) [49]. The 15-azasteroid, 1,10,11,11a-tetrahydro-11a-methyl -2H-naphtho[1,2-g]-indol-7-ol (188), inhibited the growth of mouse L—M cells and human tumour cell line, KB, at concentration of 10^{-5}M [850]. The blockage action of (188) on *in vitro* mouse fibroblast cell permeability and on respiration of isolated rat liver mitochondria has been studied [851].

The observations that there is accumulation of cholesterol in tumour cells [934,935] have prompted the studies on sterols and related analogues, with the expectation of discovering anti-neoplastic activity in such compounds [936]. 6-Aza-3,5-cholestadiene and its salts are claimed to have cytostatic effect of value in the treatment of non-malignant and malignant tumours [937]. 2-Oximino-8, 24-lanostadien-3-one [938] shows an inhibition of tumour growth of 40% in the cheekpouch test [939].

25-Azacholesterol (164) has been found to be cytostatic in tissue cultures [936]. Treatment of mice having experimental brain tumours with 20,25-diazacholesterol (163) induced an accumulation of desmosterol in the tumours but not in normal brain [940]. 6α-Dimethylaminocholestane-3β, 5α-diol, the reassigned structure [941] for the compound earlier considered to be a ring-B seco entity [942], has cytotoxic properties [943].

Since most of the effective hormonal treatments of breast carcinomas act by directly or indirectly altering the oestrogen milieau, the oestrogen antagonists (anti-oestrogens) have been of considerable interest to many clinical investigators [944]. The anti-oestrogen, epitiostanol (2*α*,3*α*-epithio-5*α*-androstan-17*β*-ol) (10275-S), inhibited not only ductal development of the mammary gland in the mouse but also the growth of an oestrogen-dependent mammary fibroadenoma in rats [945]. The anti-oestrogen caused TPDMT-4 pregnancy-dependent mammary tumours to regress [946]. The steroid has been used with some efficacy for the treatment of advanced breast cancer [947]. Mepitiostane (2*α*,3*α*-epithio-5*α*-androstan-17*β*-yl 1-methoxycyclopentyl ether) (10364-S) (92), an orally active derivative of epitiostanol, has also been tried with some advantage in advanced human carcinomas [948]. Mepitiostane inhibited the ductal growth of the mouse mammary gland and growth of hormone-dependent rat mammary tumours [421] and significantly suppressed TPDMT-4 tumours in mice [949].

As mentioned earlier, it has been suggested that danazol (127) may directly inhibit the effect of oestrogens upon endometrial cells [465]. It has been speculated that, if danazol can directly inhibit the effect of oestrogen at the endometrium, it may have new uses in the management of endometrial carcinoma and possibly other oestrogen-dependent tumours [465]. Chlormadinone acetate, a potent anti-androgen, has been used for prostatic hypertrophy and prostatic carcinoma [950]. Anabolic heterosteroid stanozolol (85) has been considered to be suitable for palliative use in the patient with active or arrested cancer [951].

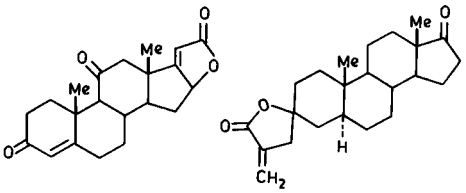
Dimethazine (96) has been tested for its effect on the immunological response of mice to Ehrlich ascites carcinoma [952]. Animals administered dimethazine alone were found to be moderately resistant to tumour take, and dimethazine in combination with immunization markedly inhibited tumour growth.

A mention may be made of 3-guanylhydrazone-androstan-17-ol which has been shown to possess a certain degree of antimitotic activity, both *in vitro* [952a,953] and *in vivo* [954]. The pteridinosteroid (209) [373] caused approximately 50% inhibition in sarcoma 180 in mice; the results of anti-tumour testing with the analogue (210) [374] were inconclusive.

The observed tumour inhibitory properties of 17*β*-acetoxy-4-androsteno [2,3-d]isoxazole and 17,17-dimethyl-18-nor-4,13(14)-androstadieno[2,3-d] isoxazole led to the synthesis of certain other [2,3-d]isoxazoles and 17,17-dimethyl-18-nor-4,13(14)-androstadieno[3,2-c]-pyrazole but there was no consistant enhancement of anti-tumour activity [290].

Of the several compounds examined, two steroidal lactones 16*β*-hydroxy-

3,11-dioxo-4,17(20)-pregnadien-21-oic acid γ-lactone (219) and 3β,16β-dihydroxy-11-oxo-5α-pregn-17(20)-en-21-oic acid γ-lactone showed potent cytotoxic activity in a growing mammalian cell culture system. These compounds have relatively low order of whole animal toxicity and endocrine activity and give good blood levels. They showed marginal inhibitory activity against S-180 and T-4 lymphoma implanted in mice.



(219) (220)

Several steroidal α-methylene-γ-lactones and related derivatives have been synthesised as potential steroid alkylating anti-tumour agents [956]. In tissue culture experiments, and preliminary *in vivo* tumour assay against Walker 256 carcinomas also, these compounds, for example (220), were active. They were inactive against both L1210 lymphoid leukemia and Ehrlich ascites carcinoma in mice.

A number of steroidal dihydro-1,3-oxazines and amines (for example, 17β-amino-5-androsten-3β-ol) have been claimed to possess certain degree of anti-tumour activity when tested in mammary carcinoma in mice [957]. 2β,16β-Dipiperidino-5α-androstane-3α,17β-diol dipivalate hydrochloride has been shown to be effective against Walker ascites in rats [958] and in tissue culture experiments [959,960].

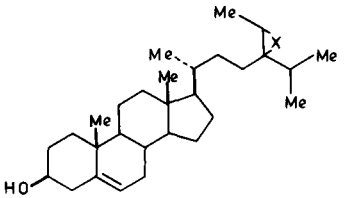
SOME MISCELLANEOUS ASPECTS

The anabolic heterosteroid furazabol (89) has shown curative effect on gastric ulcer in rats [668]. The property may be related to the original anabolic action, but the detail still remains to be studied. Furazabol also has papaverine-like property on smooth muscles. It showed non-specific inhibitory activities to the responses of guinea-pig ileum to various spasmogens, and the activities were considerably higher than those of papaverine [668].

Screening in a seroflocculation reaction against cancer and normal sera, showed 3,3-bisethylthio-5α-androstan-17β-yl acetate and 3,3-bisallylthio-5α-androstan-17β-yl acetate and 3,5-androstadien-17β-yl phenylacetate to be

highly active seroflocculating agents [961]. Study of different compounds as cholesterol gallstone dissolution rate accelerators indicates that steroidal amines and the bile acid derivatives containing the amine nitrogent or the quaternary nitrogen functional group may offer promise [962,963].

It has been suggested that 24,28-epoxyfucosterol (221) is a possible intermediate in the conversion of β-sitosterol to cholesterol in the silkworm, *Bombyx mori* [964,965]. A similar mechanism of phytosterol dealkylation which is consistent with the existence of epoxide intermediate, was also found in locust, *Locusta migratoria* [966] and yellow mealworm, *Tenebrio molitor* [967]. The steroidal aziridine 24,28-iminofucosterol (222) disrupted the normal growth and development of larvae of the silkworm, *B. mori* and was found to be potent inhibitor of dealkylation of β-sitosterol [968].



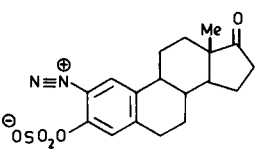
(221) X = -O-

(222) X = -N(H)-

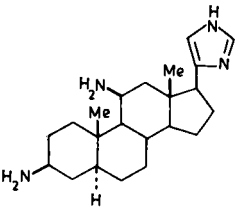
4-Acetatomercuri-17β-estradiol (referred to as 4-mercuri-17β-estradiol) has been prepared and studied for its reaction with sulphydryl compounds and proteins [969]. The experimental observations are compatible with a mechanism in which steroid moiety of the compound delivers mercury to the allosteric binding site for 17β-estradiol. 4-Mercuri-17β-estradiol may be used for identification of high affinity present in steroid-converting enzymes and in receptor proteins of target organs. Binding of 4-mercury-17β-estradiol to the cytoplasmic receptor protein of immature rat uterus has been demonstrated; pretreatment with 17β-estradiol abolished this binding.[970].

To extend study of the specificity to include other amino acid residues, 2-diazoestrone sulphate (223) has been examined in affinity labelling experiments [971]. Under the physiological conditions, 2-diazoestrone sulphate couples with tryptophan, cysteine and histidine (and their various esters and peptides) and as such could be useful in characterizing steroid binding sites containing any of these three reactive amino acid residues.

The steroid nucleus provides a large rigid framework for biomimetic type of catalytic studies. The water-soluble heterosteroid (224) [972] has a potentially



(223)



(224)

catalytic substituent. This enzyme model system acts as a catalyst for the hydrolysis of active esters, with a definite specificity for esters of 3-arylpropionic acid [973]. It is concluded that imidazole-catalysed hydrolysis of esters is facilitated by hydrophobic interactions between substrate and catalyst.

Several bis-heterosteroids derived from conessine or cholic acid solubilise perylene in aqueous solution without evidence of micelle formation, and cause spectral changes in aqueous pinacyanol iodide; monoheterosteroids examined show these effects (characteristic of hydrophobic interaction) only on micellisation or not at all [974].

Interaction of diamino steroids (derivatives of 5α-pregnane, 5-pregnene, and 5-androstene) with DNA has been studied [975—977]. Three types of DNA-steroid complexes were formed, *i.e.*, one in which a net stabilization of the helical structure is observed, a second in which the DNA helix is extensively destabilised by the presence of steroidal micelles on the surface of the helix, and a third in which the steroidal micelles are organised along the phosphate residue of the denatured form. An examination of the interaction of different 3,17-diamino-5α-androstane derivatives with nucleic acid helices indicate that the steroidal amines selectively stabilise the DNA helical structures, while causing the ribose-containing acids to unravel and denature [978,979].

REFERENCES

1. Y. Sato, in: Chemistry of the Alkaloids, ed. S.W. Pelletier (Van Nostrand Reinhold Company, New York, 1970) p. 591.
2. K.S. Brown, Jr., in Ref. 1, p. 631.
3. G.G. Habermehl, in: MTP International Review of Science; Organic Chemistry, Series 1: Vol. IX: Alkaloids, ed. K. Wiesner (Butterworths, London, 1973) p. 235.
4. F. Khoung-Huu-Lainé and R. Goutarel, in: The Alkaloids, Sen. Reporter J.E. Saxton (Chemical Society, London, 1974) Vol. IV, p. 346.

5. V. Prelog and O. Jeger, in: The Alkaloids, eds. R.H.F. Manske and H.L. Holmes (Academic Press, New York, 1953) Vol. III, p. 247.
6. V. Prelog and O. Jeger, in Ref. 5, Vol. VII (1960) p. 343.
7. K. Schreiber, in Ref. 5, Vol. X (1968) p. 1.
8. R.B. Herbert, in Ref. 4, p. 383.
9. O. Jeger and V. Prelog, in Ref. 5, Vol. VII (1960) p. 363.
10. S.M. Kupchan and A.W. By, in Ref. 5, Vol. X (1968) p. 193.
11. J. Tomko and Z. Votický, in Ref. 5, Vol. XIV (1973) p. 1.
12. O. Jeger, and V. Prelog, in Ref. 5, Vol. VII (1960) p. 319.
13. V. Cerny and F. Sorm, in Ref. 5, Vol. IX (1967) p. 305.
14. T. Tokuyama, J. Daly, B. Witkop, I.L. Karle and J. Karle, J. Am. Chem. Soc., 90 (1968) 1917.
15. T. Tokuyama, J. Daly and B. Witkop, J. Am. Chem. Soc., 91 (1969) 3931.
16. R. Imhof, E. Gössinger, W. Graf, L. Berner-Fenz, H. Berner, R. Schaufelberger and H. Wehrli, Helv. Chim. Acta, 56 (1973) 139.
17. E.X. Albuquerque, J.W. Daly and B. Witkop, Science, 172 (1971) 995.
18. T. Narahashi, Physiol. Rev. (1974) 813.
19. E.X. Albuquerque, I. Seyama and T. Narahashi, J. Pharmacol. Exp. Ther., 184 (1973) 308.
20. E.X. Albuquerque, J.E. Warnick, F.M. Sansone and J. Daly, J. Pharmacol. Exp. Ther., 184 (1973) 315.
21. J.E. Warnick, E.X. Albuquerque, R. Onur, S.E. Jansson, J. Daly, T. Tokuyama and B. Witkop, J. Pharmacol. Exp. Ther., 193 (1975) 232.
22. G.S. Shotzberger, E.X. Albuquerque and J.W. Daly, J. Pharmacol. Exp. Ther., 196 (1976) 433.
23. P. Honerjäger and M. Reiter, Br. J. Pharmacol., 58 (1976) 415P.
24. E.X. Albuquerque, N. Brookes, R. Onur and J.E. Warnick, Mol. Pharmacol., 12 (1976) 82.
25. G. Habermehl, in Ref. 5, Vol. IX (1967) p. 427.
26. G. Habermehl, in: Prog. Org. Chem., 7 (1968) 35.
27. S. Hara and K. Oka, J. Am. Chem. Soc., 89 (1967) 1041.
28. K. Oka and S. Hara, Tetrahedron Lett., (1969) 1189, 1193.
29. G. Habermehl and A. Haaf, Justus Liebig's Ann. Chem., 722 (1969) 155.
30. G. Habermehl and A. Haaf, Z. Naturforsch., 23b (1968) 1551.
31. K. Oka and S. Hara, J. Am. Chem. Soc., 99 (1977) 3859.
32. R. Binder and H. Wehrli, Helv. Chim. Acta, 51 (1968) 1989.
33. K. Oka and S. Hara, Tetrahedron Lett., (1969) 1987.
34. K. Oka, Y. Ike and S. Hara, Tetrahedron Lett., (1969) 4543, 4547.
35. G. Habermehl and A. Haaf, Z. Naturforsch., 24b (1969) 1414.
36. G. Habermehl and A. Haaf, Z. Naturforsch., 25b (1970) 191.
37. Y. Shimizu, Tetrahedron Lett., (1972) 2919.
38. M.H. Benn and R. Shaw, J. Chem. Soc. Chem. Commun. (1973) 288.
39. K.H. Michel, R.L. Hamill, S.H. Larsen and R.H. Williams, J. Antibiot., 28 (1975) 102.
40. S. Neidle, D. Rogers and M.B. Hursthouse, J. Chem. Soc. Perkin II (1972) 760.
41. J. MacMillan, T.J. Simpson and S.K. Yeboah, J. Chem. Soc. Chem. Commun. (1972) 1063.
42. K.K. Chen and A. Kovarikova, J. Pharm. Sci., 56 (1967) 1535.

43. H.O. Huisman, in: MTP International Review of Science, ed. W.F. Johns (Butterworths, London, 1973) Vol. VIII, p. 235.
44. L. Tökés, in: Steroid Reactions, ed. C. Djerassi (Holden-Day, San Francisco, 1963) p. 457.
45. I. Ninomiya, Yuki Gosei Kageku Kyokai Shi, 30 (1972) 318.
46. R. Pappo, Intra-Science Chem. Rep., 3 (1969) 105.
47. S.R. Ramdas and A.P. Chaudhuri, J. Sci. Ind. Res., 34 (1975) 563.
48. R. Pappo and R.J. Chorvat, Tetrahedron Lett., (1972) 3237.
49. R.J. Chorvat and R. Pappo, J. Org. Chem., 41 (1976) 2864.
50. R.J. Chorvat and R. Pappo, Tetrahedron Lett., (1975) 623.
51. W.N. Speckamp, H. de Koning, U.K. Pandit and H.O. Huisman, Tetrahedron, 21 (1965) 2517.
52. J.H. Burckhalter and H. Watanabe, Chem. Eng. News, (1963) 40.
53. R.E. Brown, D.M. Lustgarten, R.J. Stanaback and R.I. Meltzer, J. Org. Chem., 31 (1966) 1489.
54. R. Clarkson, J. Chem. Soc., (1965) 4900.
55. R.W. Kierstead, A. Faraone and A. Boris, J. Med. Chem., 10 (1967) 177.
56. J.S. Baran, J. Med. Chem., 10 (1967) 1039.
57. S. Rakhit and M. Gut, Steroids, 4 (1964) 291.
58. R. Pappo and C.J. Jung, Tetrahedron Lett., (1962) 365.
59. R. Hirschmann, N.G. Steinberg, and R. Walker, J. Am. Chem. Soc., 84 (1962) 1270.
60. H. Smith, G.H. Douglas and C.R. Walk, Experientia, 20 (1964) 418.
61. Ch. R. Engel, R.C. Rastogi and M.N. Roy Chowdhury, Steroids, 19 (1972) 1.
62. Ch. R. Engel, D. Mukherjee, M.N. Roy Chowdhury, G. Ramani and V.S. Salvi, J. Steroid Biochem., 6 (1975) 585.
63. V.S. Salvi, D. Mukherjee, M.N. Roy Chowdhury and Ch. R. Engel, Steroids, 27 (1976) 717.
64. P. Rosen and G. Oliva, J. Org. Chem., 38 (1973) 3040.
65. W.N. Speckamp, J.G. Westra and H.O. Huisman, Tetrahedron, 26 (1970) 2353.
66. G. Zanati and M.E. Wolff, J. Med. Chem., 15 (1972) 368.
67. G. Zanati, G. Gaare and M.E. Wolff, J. Med. Chem., 17 (1974) 561.
68. C.H. Chen and K.D. Berlin, Phosphorus, 1 (1971) 49.
69. Y. Kashman and O. Awerbouch, Tetrahedron Lett., (1973) 3217.
70. G.I. Zhungietu and G.N. Dorofeenko, Russ. Chem. Rev., 36 (1967) 24.
71. A.A. Akhrem and Yu. A. Titov, Russ. Chem. Rev., 36 (1967) 311.
72. H. Singh, V.V. Parashar, R.B. Mathur and R.K. Malhotra, Indian J. Pharm., 34 (1972) 1.
73. M. Alauddin and M. Martin-Smith, J. Pharm. Pharmacol., 14 (1962) 325.
74. M. Alauddin and M. Martin-Smith, J. Pharm. Pharmacol., 14 (1962) 469.
75. M. Martin-Smith and M.F. Sugrue, J. Pharm. Pharmacol., 16 (1964) 569.
76. H. Singh, S. Padmanabhan and V.V. Parashar, J. Inst. Chemists (India), 39 (1967) 54.
77. H. Singh, V.V. Parashar, S. Padmanabhan and R.B. Mathur, Indian J. Pharm. Ed., 4(2) (1970) 2.
78. W.R. Buckett, Adv. Steroid Biochem. Pharmacol., 3 (1972) 39.
79. C.M. Cimarusti, F.F. Giarrusso, P. Grabowich and S.D. Levine, Steroids, 26 (1975) 359.
80. Z.A. Zderic, O. Halpern, H. Carpio, A. Ruiz, D.C. Limon, L. Magaña, H. Jiménez, A. Bowers and H.J. Ringold, Chem. Ind. (London), (1960) 1625.

81. R. Hirschmann, N.G. Steinberg, P. Buchschacher, J.H. Fried, G.J. Kent, M. Tishler and S.L. Steelman, J. Am. Chem. Soc., 85 (1963) 120.
82. J.H. Fried, H. Mrozik, G.E. Arth, T.S. Bry, N.G. Steinberg, M. Tishler, R. Hirschmann and S.L. Steelman, J. Am. Chem. Soc., 85 (1963) 236.
83. R. Hirschmann, P. Buchschacher, N.G. Steinberg, J.H. Fried, R. Ellis, G.J. Kent and M. Tishler, J. Am. Chem. Soc., 86 (1964) 1520.
84. R. Hirschmann, N.G. Steinberg, E.F. Schoenewaldt, W.J. Paleveda and M. Tishler, J. Med. Chem., 7 (1964) 352.
85. R.G. Strachan, N.G. Steinberg, M. Tishler and R. Hirschmann, J. Med. Chem., 7 (1964) 355.
86. S.L. Steelman, E.R. Morgan and M.S. Glitzer, Steroids, 18 (1971) 129.
87. I.T. Harrison, C. Beard, L. Kirkham, B. Lewis, I.M. Jamieson, W. Rooks and J.H. Fried, J. Med. Chem., 11 (1968) 868.
88. J. Hannah, K. Kelly, A.A., Patchett, S.L. Steelman and E.R. Morgan, J. Med. Chem., 18 (1975) 168.
89. H. Mrozik, P. Buchschacher, J. Hannah and J.H. Fried, J. Med. Chem., 7 (1964) 584.
90. K. Ueno, K. Obata and M. Shimizu, Chem. Pharm. Bull., 15 (1967) 523.
91. J.H. Fried, P. Buchschacher and H. Mrozik, Steroids, 2 (1963) 399.
92. E. Diamanti and G.E. Bianchi, Arzneim., Forsch., 21 (1971) 251.
93. E.T. Ordoñez, Arzneim. Forsch., 21 (1971) 248.
94. V. Galli and C. Esposito, Rass. Dermatol. Siplogr., 26 (1973) 37.
95. E.T. Ordoñez, Arzneim. Forsch., 24 (1974) 2018.
96. E.T. Ordoñez, Arzneim. Forsch., 24 (1974) 2015.
97. G. Nathansohn, G. Winters and E. Testa, J. Med. Chem., 10 (1967) 799.
98. G. Nathansohn, C.R. Pasqualucci, P. Radaelli, P. Schiatti, D. Selva and G. Winters, Steroids, 13 (1969) 365.
99. J.D. Lewis, B.D. Cameron, D.R. Hawkins, L.F. Chasseaud and E.R. Franklin, Arzneim. Forsch., 25 (1975) 1646.
100. G. Nathansohn, G. Odasso and S. Ceriani, 3rd Int. Congr. Hormonal Steroids, Excerpta Medica, No. 210, Abstr. 364 (1970).
101. G. Winters, M. Cannas and G. Nathansohn, 3rd Int. Congr. Hormonal Steroids, Excerpta Medica, No. 210, Abstr. 171 (1970).
102. J.E. Pike, G. Slomp and F.A. MacKellar, J. Org. Chem., 28 (1963) 2502.
103. F. Kagan, R.D. Birkenmeyer and B.J. Magerlein, J. Med. Chem., 7 (1964) 751.
104. Y.F.J. Choo-Kang, E.J. Cooper, A.E. Tribe and I.W.B. Grant, Br. J. Dis. Chest, 66 (1972) 101.
105. H.M. Brown, G. Storey and W.H.S. George, Br. Med. J., 1 (1972) 585.
106. J. Lovera, D.M. Cooper, C. Collins-Williams, H. Levison, J.D. Bailey and R.P. Orange, J. Allergy Clin. Immun., 57 (1976) 112.
107. D.A. Hussar, Am. J. Pharm., 149 (1977) 5.
108. A. Bianchetti, G. Bossoni, L. Guarneri, L. Giudici and M. Riva, Arzneim. Forsch., 27 (II) (1977) 2096.
109. C.G. Bergstrom, J. Med. Chem., 11 (1968) 875.
110. Y. Nagai, Chem. Pharm. Bull., 17 (1969) 1749.
111. G. Teutsch, L. Weber, G. Page, E.L. Shapiro, H.L. Herzog, R. Neri and E.J. Collins, J. Med. Chem., 16 (1973) 1370.
112. H.M. Kissman, R.E. Schaub and M.J. Weiss, J. Med. Chem., 10 (1967) 252.
113. K. Takeda and T. Komeno, Chem. Pharm. Bull., 8 (1960) 468.

114. T. Kawasaki and E. Mosettig, J. Org. Chem., 27 (1962) 1374.
115. K. Takeda, T. Kubota and J. Kawanami, Chem. Pharm. Bull., 8 (1960) 615.
116. G.B. Fregnan and G.K. Suchowsky, Eur. J. Pharmacol., 3 (1968) 251.
117. J.R.J. Sorenson, Inflammation, 1 (1976) 317.
118. J.R.J. Sorenson, J. Med. Chem., 19 (1976) 135.
119. G. Engelhardt, Arzneim. Forsch., 13 (1963) 588.
120. G. Engelhardt, Therapiewoche, 25 (1975) 5867, 5870, 5875.
121. H.D. Brown, A.R. Matzuk, D.R. Hoff and L.H. Sarett, J. Org. Chem., 27 (1962) 961.
122. F. Hudoson, O. Mantsch, I. Jude, D. Breazu, B. Cuparencu and N. Sterescu, Arzneim. Forsch., 19 (1969) 684.
123. M. Heller, R.H. Lenhard and S. Bernstein, Steroids, 5 (1965) 615.
124. C.M. Cimarusti, S.T. Chao and L.J. Brannick, J. Med. Chem., 19 (1976) 721.
125. L. Toscano, L. Barlotti, G. Fioriello, G. Grisanti, G. Bossoni, L.M. Biraschi and M. Riva, Arzneim. Forsch., 27 (II) (1977) 1628.
126. C.G. Sparkes and L. Wilson, Br. J. Dermatol., 90 (1974) 197.
127. F.K. Bagatell and M.A. Augustine, Curr. Ther. Res., 16 (1974) 748.
128. E. Leibsohn and F.K. Bagatell, Br. J. Dermatol., 90 (1974) 435.
129. R. Clayton, Br. J. Dermatol., 96 (1977) 71.
130. M. Ganguly and J.C. Warren, J. Biol. Chem., 246 (1971) 3646.
131. L.L. Smith, M. Marx, H. Mendelsohn, T. Foell and J.J. Goodman, J. Am. Chem. Soc., 84 (1962) 1265.
132. N.R. Stephenson, Acta Endocrinol., 36 (1961) 287.
133. R.I. Dorfman, A.S. Dorfman, E.J. Agnello, S.K. Figdor and G.D. Laubach, Acta Endocrinol., 37 (1961) 343.
134. L. Szporny, L. Dömök, P. Görög and G. Fekete, Steroids, 7 (1966) 181.
135. P. Görög and L. Szporny, J. Pharm. Pharmacol., 17 (1965) 250.
136. Gy. T. Hajós, L. Szporny and Z. Tuba, Steroids, 15 (1970) 449.
137. T. Fehér, O. Koref, A. Tonkó, L. Bodrogi and E. Poteczin, Arzneim. Forsch., 25 (1975) 1314.
138. D.R. Torossian, G.G. Aubard and J.M.G.A. Legeai, Ger. Patent 2,357,778; Chem. Abstr., 82 (1975) 156584.
139. W.J. Leanza, J.P. Conbere, E.F. Rogers and K. Pfister 3rd, J. Am. Chem. Soc., 76 (1954) 1691.
140. J. Schmidlin and L. Ehmann, Swiss Patent 543,494; Chem. Abstr., 80 (1974) 60083.
141. E.J. Agnello and P.R. Farina, J. Med. Chem., 15 (1972) 363.
142. H. Laurent, E. Gerhards and R. Wiechert, Angew. Chem. Int. Ed. Engl., 14 (1975) 65.
143. H. Laurent, E. Gerhards and R. Wiechart, Arzneim. Forsch., 27 (II) (1977) 2187.
144. H.-J. Heite and J. Etspiller, Arzneim. Forsch., 27 (II) (1977) 2234.
145. R. Reckers, Arzneim. Forsch., 27(II) (1977) 2240.
146. R.W. Kierstead, A. Faraone and M.W. Goldberg, J. Med. Chem., 6 (1963) 77.
147. A. Ercoli and R. Gardi, U.S. Patent 3,789,121; Chem. Abstr., 80 (1974) 96228.
148. G. Anner and H. Kaufmann, Ger. Patent 2,451,777; Chem. Abstr., 83 (1975) 97727.
149. E. Bajusz and G. Jasmin, Rev. Can. Biol., 20 (1961) 829.
150. R.B. Filderman and B.A. Kovacs, Br. J. Pharmacol., 37 (1969) 748.
151. R.H. Wilson, G.W. Poley and F. De Eds, Toxicol. Appl. Pharmacol., 3 (1961) 39.
152. N.I. Skachkova, A.D. Turova and A.F. Końkova, Med. Zh. Uzbekistana (2) (1974) 55; Chem. Abstr., 81 (1974) 21060.
153. D. Wiesinger, H.U. Gubler, W. Haefliger and D. Hauser, Experientia, 30 (1974) 135.

154. L.L. Smith, Steroids, 9 (1967) 289.
155. L. Szporny and C. Mészáro, Acta Physiol., 21 (1962) 359.
156. G.W. Barber and M. Ehrenstein, J. Org. Chem., 26 (1961) 1230.
157. M.E. Wolff, D. Feldman, P. Catsoulacos, J.W. Funder, C. Hancock, Y. Amano and I.S. Edelman, Biochemistry, 14 (1975) 1750.
158. H.C. Neumann, G.O. Potts, W.T. Ryan and F.W. Stonner, J. Med. Chem., 13 (1970) 948.
159. R.A. Ferrari and A. Arnold, Biochem. Biophys. Acta, 77 (1963) 349, 357.
160. A.S. Goldman, W.C. Yakovac and A.M. Bongiovanni, Endocrinology, 77 (1965) 1105.
161. A.M. Neville and L.L. Engel, J. Clin. Endocrinol. Metab., 28 (1968) 49.
162. A.S. Goldman, A.M. Bongiovanni and W.C. Yakovac, Proc. Soc. Exp. Biol. Med., 121 (1966) 757.
163. R.V. Farese, Steroids, 15 (1970) 245.
164. H.R. Harding and G.O. Potts, Excerpta Medica Int. Congr. Series, No. 99, Abstr. 345 (1965).
165. J.A. Cella and C.M. Kagawa, J. Am. Chem. Soc., 79 (1957) 4808.
166. C.M. Kagawa, J.A. Cella and C.G. Van Arman, Science, 126 (1957) 1015.
167. J.A. Cella and R.C. Tweit, J. Org. Chem., 24 (1959) 1109.
168. H.J. Hess, J. Org. Chem., 27 (1962) 1096.
169. K. Heusler, Helv. Chim. Acta, 45 (1962) 1939.
170. G.E. Arth, H. Schwam, L.H. Sarett and M. Glitzer, J. Med. Chem., 6 (1963) 617.
171. D. Bertin and J. Perronnet, Bull. Soc. Chim. Fr. (1964) 564.
172. W.F. Johns and E.A. Brown, J. Org. Chem., 31 (1966) 2099.
173. J.A. Cella, E.A. Brown and R.R. Burtner, J. Org. Chem., 24 (1959) 743.
174. A.A. Patchett, F. Hofmann, F.F. Giarrusso, H. Schwam and G.E. Arth, J. Org. Chem., 27 (1962) 3822.
175. L.N. Nysted and R.R. Burtner, J. Org. Chem., 27 (1962) 3175.
176. E. Farkas and J.A. Swallow, J. Med. Chem., 7 (1964) 739.
177. G.W. Moersch and P.L. Creger, J. Heterocycl. Chem., 2 (1965) 207.
178. H.G. Lehmann, Angew. Chem. Int. Ed. Engl., 4 (1965) 783.
179. N.H. Dyson, J.A. Edwards and J.H. Fried, Tetrahedron Lett., (1966) 1841.
180. G.W. Moersch, D.E. Evans and G.S. Lewis, J. Med. Chem., 10 (1967) 254.
181. R.K. Sharma, N.J. Doorenbos and N.S. Bhacca, J. Pharm. Sci., 60 (1971) 1677.
182. A.A. Patchett, U.S. Patent 3,257,390 (1966); Chem. Abstr., 65 (1966) 9001.
183. B. Patelli and R. Sciaky, Fr. Patent 1,384,204 (1965); Chem. Abstr., 62 (1965) 14789.
184. D.F. Morrow, R.M. Hofer and T.P. Culbertson, J. Med. Chem., 10 (1967) 133.
185. G. Roussean and V. Torelli, Ger. Offen 2,541,659 (1976); Chem. Abstr. 85 (1976) 46951.
186. L.J. Chinn, J. Med. Chem., 9 (1966) 602.
187. P. Crabbé, J. Revilla and A. Bowers, J. Med. Chem., 6 (1963) 182.
188. P.D. Klimstra, J. Med. Chem., 10 (1967) 503.
189. R.C. Tweit, F.B. Colton, N.L. McNiven and W. Klyne, J. Org. Chem., 27 (1962) 3325.
190. G.R. Lenz, U.S. Patent 3,926,962 (1975); Chem. Abstr., 84 (1976) 122150.
191. R.M. Weier and L.M. Hofmann, J. Med. Chem., 18 (1975) 817.
192. R.M. Weier and L.M. Hofmann, J. Med. Chem., 20 (1977) 1304.
193. R.M. Weier, Ger. Patent 2,443,746 (1975); Chem. Abstr., 83 (1975) 28458.
194. R.M. Weier and L.M. Hofmann, J. Med. Chem., 19 (1976) 975.
195. W.F. Johns, 166th Nat. Meeting Am. Chem. Soc. Chicago (1973) I11.
196. A. Karim and E.A. Brown, Steroids, 20 (1972) 41.

197. L.J. Chinn and B.N. Desai, J. Med. Chem., 18 (1975) 268.
198. L.M. Hofmann, L.J. Chinn, H.A. Pedrera, M.I. Krupnick and O.D. Suleymanov, J. Pharmacol. Exp. Ther., 194 (1975) 450.
199. E.A. Brown, R.D. Muir and J.A. Cella, J. Org. Chem., 25 (1960) 96.
200. E.A. Brown and R.R. Burtner, J. Med. Chem., 6 (1963) 732.
201. R.C. Tweit, E.A. Brown, S. Kraychy, S. Mizuba, R.D. Muir and R.T. Nicholson, Chem. Pharm. Bull., 12 (1964) 859.
202. L.J. Chinn and L.M. Hofmann, J. Med. Chem., 16 (1973) 839.
203. J. Hora, Collect. Czech. Chem. Commun., 30 (1965) 70.
204. A. Kasal and V. Černý, Collect. Czech. Chem. Commun., 31 (1966) 2759.
205. L.L. Smith and D.M. Teller, J. Med. Chem., 7 (1964) 531.
206. M.E. Wolff and W. Ho, J. Med. Chem., 7 (1964) 681.
207. J.W. Hollifield, T.J. McKenna, J. McD. Wolff and G.W. Liddle, Clin. Res., 23 (1975) 12 A.
208. C.M. Kagawa, Endocrinology, 67 (1960) 125.
209. C.L. Gantt, N.Y. J. Med., 61 (1961) 756.
210. D.H.P. Streeten, Clin. Pharmacol. Ther., 2 (1961) 359.
211. J.A. Snell and E.P. Cordner, Med. J. Aust., 1 (1977) 828.
212. G.V. Brown, W.A. Liston and D.S.K. Brookfield, Lancet, 1 (1977) 855.
213. N. Gochman and C.L. Gantt, J. Pharmacol. Exp. Ther., 135 (1962) 312.
214. L. Zicha, F. Weist, F. Scheiffarth and E. Schmid, Arzneim. Forsch., 14 (1964) 699.
215. A. Karim, J. Zagarella, J. Hribar and M. Dooley, Clin. Pharmacol. Ther., 19 (1976) 158.
216. W. Sadée, M. Dagcioglu and R. Schröder, J. Pharmacol. Exp. Ther., 185 (1973) 686.
217. A. Karim, J. Zagarella, T.C. Hutsell and M. Dooley, Clin. Pharmacol. Ther., 19 (1976) 177.
218. B. Solymoss, S. Tóth, S. Varga and M. Krajny, Steroids, 16 (1970) 263.
219. W. Sadée, M. Dagcioglu and S. Riegelman, J. Pharm. Sci., 61 (1972) 1126.
220. W. Sadée, U. Abshagen, C. Finn and N. Rietbrock, Naunyn-Schmiedeberg's Arch. Pharmacol., 283 (1974) 303.
221. A. Karim, C. Kook, D. Zitzewitz, J. Zagarella, M. Doherty and J. Campion, Drug Metab. Dispos., 4 (1976) 547.
222. A. Karim, J. Hribar, M. Doherty, W. Aksamit, D. Chappelow, E. Brown, C. Markos, L.J. Chinn, D. Liang and J. Zagarella, Xenobiotica, 7 (1977) 585.
223. U. Abshagen, H. Rennekamp, K. Koch, M. Senn and W. Steingross, Steroids, 28 (1976) 467.
224. U. Abshagen, H. Rennekamp and G. Luszpinski, Naunyn-Schmiedberg's Arch. Pharmacol., 296 (1976) 37.
225. A. Karim, J. Hribar, W. Aksamit, M. Doherty and L.J. Chinn, Drug Metab. Dispos., 3 (1975) 467.
226. L.J. Chinn, E.A. Brown, S.S. Mizuba and A. Karim, J. Med. Chem., 20 (1977) 352.
227. C.M. Kagawa, D.J. Bouska, M.L. Anderson and W.F. Krol, Arch. Int. Pharmacodyn. Ther., 149 (1964) 8.
228. H.C. Erbler, H. Wernze and M. Hilfenhaus, Eur. J. Clin. Pharmacol., 9 (1976) 253.
229. L. Ramsay, M. Asbury, J. Shelton and I. Harrison, Clin. Pharmacol. Ther., 21 (1977) 602.
230. L.E. Ramsay, J.R. Shelton, D. Wilkinson and M.J. Tidd, Br. J. Clin. Pharmacol., 3 (1976) 607.

231. A.M. Finn, C. Finn and W. Sadée, Res. Commun. Chem. Path. Pharmacol., 15 (1976) 613.
232. L. Ramsay, J. Shelton, I. Harrison, M. Tidd and M. Asbury, Clin. Pharmacol. Ther., 20 (1976) 167.
233. U. Abshagen, H. Rennekamp and G. Luszpinski, Eur. J. Clin. Pharmacol., 11 (1977) 169.
234. U. Abshagen, U. von Grodzicki, U. Hirschberger and H. Rennekamp, Naunyn-Schmiedberg's Arch. Pharmacol., 300 (1977) 281.
235. H.C. Erbler, Naunyn-Schmiedeberg's Arch. Pharmacol., 285 (1974) 395.
236. H.C. Erbler, Naunyn-Schmiedeberg's Arch. Pharmacol., 286 (1974) 145.
237. H.C. Erbler, Naunyn-Schmiedeberg's Arch. Pharmacol., 277 (1973) 139.
238. H.C. Erbler, Naunyn-Schmiedeberg's Arch. Pharmacol., 273 (1972) 366.
239. H.C. Erbler, Naunyn-Schmiedeberg's Arch. Pharmacol., 280 (1973) 331.
240. J.W. Greiner, R.E. Kramer, J. Jarrell and H.D. Colby, J. Pharmacol. Exp. Ther., 198 (1976) 709.
241. M.G. Crane and J.J. Harris, Am. J. Med. Sci., 260 (1970) 311.
242. R.F. Spark and J.C. Melby, Ann. Int. Med., 75 (1971) 831.
243. R.M. Carey, J.G. Douglas, J.R. Schweikert and G.W. Liddle, Arch. Int. Med., 130 (1972) 849.
244. M.J. Kert, L.W. Tarr, S. Franklin, E. Gold, R. Akun and W. Maxwell, Angiology, 23 (1972) 617.
245. S.-E. Fagerberg and K. Göransson, Lakartidningen, 71 (1974) 175.
246. F. Klumpp, B. Braun, D. Klaus, R. Lemke, J. Zehner and P. Zöfel, Dtsch. Med. Wschr., 100 (1975) 577.
247. B.I. Hoffbrand, C.J. Edmonds and T. Smith, Brit. Med. J., 1 (1976) 682.
248. R.K. Ferguson, D.M. Turek and D.R. Rovena, Clin. Pharmacol. Ther., 21 (1977) 62.
249. B.E. Karlberg, B. Kagedal, L. Tegler and K. Tolagen, Br. Med. J., 1 (1976) 251.
250. B.E. Karlberg, B. Kagedal, L. Tegler, K. Tolagen and B. Bergman, Am. J. Cardiol., 37 (1976) 642.
251. R. Schröder, B. Ramdohr, U. Hüttemann and K. Schüren, Dtsch. Med. Wschr., 97 (1972) 1535.
252. A. Baethmann, W. Land, K. Koczorch, P. Stass and W. Brendel, Eur. Surg. Res., 1 (1969) 212.
253. K. Koczorek, Med. Klin., 66 (1971) 1583.
254. E. Freerksen, Internist. Parax., 13 (1973) 589.
255. M. Fox, A.S. Minot and G.W. Liddle, J. Clin. Endocrinol. Metab., 22 (1962) 921.
256. T.S. Danowski, F.A. Lee, R.F. Cohn, R.D. D'Ambrosia and N.R. Limaye, Am. J. Dis. Child., 109 (1965) 526.
257. W. Metcalf, H. Blumberg and J. Roach, Metabolism, 14 (1965) 59.
258. D.C. Moore, D.S. Tattoni, R.H.A. Ruvalcaba, G.A. Limbeck and V.C. Kelley, J. Pediatr., 90 (1977) 462.
259. J. Geller, Acta Endocrinol., 59 (1968) 307.
260. G.A. Limbeck, R.H.A. Ruvalcaba, C.P. Mahoney and V.C. Kelley, Clin. Pharmacol. Ther., 12 (1971) 798.
261. S. Raiti, E. Trias, L. Levitsky and M.S. Grossman, Am. J. Dis. Child., 126 (1973) 597.
262. A.L. Rosenbloom and J.L. Frias, Am. J. Dis. Child., 125 (1973) 385.
263. A. Karim, R.E. Ranney, J. Zagarella and H.I. Maibach, Clin. Pharmacol. Ther., 14 (1973) 862.

264. E.F. Nutting and D.W. Calhoun, Endocrinology, 84 (1969) 441.
265. N.W. Atwater and J.W. Ralls, J. Am. Chem. Soc., 82 (1960) 2011.
266. R.W. Scot Skinner, R.V. Pozderac, R.E. Counsell and P.A. Weinhold, Steroids, 25 (1975) 189.
267. A.P. Shroff and C.H. Harper, J. Med. Chem., 12 (1969) 190.
268. A.P. Shroff, J. Pharm. Sci., 59 (1970) 110.
269. N.J. Doorenbos and C.L. Huang, J. Org. Chem., 26 (1961) 4106.
270. M.E. Wolff and G. Zanati, J. Med. Chem., 12 (1969) 629.
271. M.E. Wolff, G. Zanati, G. Shanmugasundarum, S. Gupte and G. Aadahl, J. Med. Chem., 13 (1970) 531.
272. S.D. Levine, J. Med. Chem., 8 (1965) 537.
273. J.A. Vida and A. Gut, J. Med. Chem., 6 (1963) 792.
274. G.R. Pettit and T.R. Kasturi, J. Med. Chem., 13 (1970) 1244.
275. G. Zanati and M.E. Wolff, J. Med. Chem., 14 (1971) 958.
276. M.E. Wolff and G. Zanati, Experientia, 26 (1970) 1115.
277. G. Zanati and M.E. Wolff, J. Med. Chem., 14 (1971) 961.
278. R.W. Guthrie, A. Boris, J.G. Mullin, F.A. Mennona and R.W. Kierstead, J. Med. Chem., 16 (1973) 257.
279. J. Bowler, R. Clarkson and P. Doyle, J. Chem. Soc. (1968) 2111.
280. C.G. Pitt, A.E. Friedman, D. Rector and M.C. Wani, Tetrahedron, 31 (1975) 2369.
281. R.O. Clinton, A.J. Manson, F.W. Stonner, A.L. Beyler, G.O. Potts and A. Arnold, J. Am. Chem. Soc., 81 (1959) 1513.
282. R.O. Clinton, A.J. Manson, F.W. Stonner, H.C. Neumann, R.G. Christiansen, R.L. Clarke, J.H. Ackerman, D.F. Page, J.W. Dean, W.B. Dickinson and C. Carabateas, J. Am. Chem. Soc., 83 (1961) 1478.
283. R.O. Clinton, R.L. Clarke, F.W. Stonner, A.J. Manson, K.F. Jennings and D.K. Phillips, J. Org. Chem., 27 (1962) 2800.
284. M.L. Tainter, A. Arnold, A.L. Beyler, F. Coulston, G.O. Potts and C.H. Roth, Clin. Med., 71 (1964) 2089.
285. H. Keneko, K. Nakamura, Y. Yamato and M. Kurokawa, Chem. Pharm. Bull., 17 (1969) 11.
286. T. Nambara, K. Shimada, S. Iwamura, M. Mori and M. Nokubo, Chem. Pharm. Bull., 21 (1973) 2474.
287. R.O. Clinton, A.J. Manson, F.W. Stonner, R.G. Christiansen, A.L. Beyler, G.O. Potts and A. Arnold, J. Org. Chem., 26 (1961) 279.
288. A.J. Manson, F.W. Stonner, H.C. Neumann, R.G. Christiansen, R.L. Clarke, J.H. Ackerman, D.F. Page, J.W. Dean, D.K. Phillips, G.O. Potts, A. Arnold, A.L. Beyler and R.O. Clinton, J. Med. Chem., 6 (1963) 1.
289. E. Caspi and D.M. Piatak, Chem. Ind. (London) (1962) 1984.
290. E. Caspi and D.M. Piatak, Can. J. Chem., 41 (1963) 2294.
291. K.E. Fahrenholtz, K.P. Meyers and R.W. Kierstead, J. Med. Chem., 15 (1972) 1056.
292. A.-A. Shishkina, T.I. Ivanenko, V.M. Rzheznikov and K.K. Pivnitskii, Khim-Farm. Zh., 8 (1974) 7; Chem. Abstr., 80 (1974) 96203.
293. G. Ohta, K. Koshi and K. Obata, Chem. Pharm. Bull., 16 (1968) 1487.
294. J.A. Zderic, H. Carpio, A. Ruiz, D.C. Limon, F. Kincl and H.J. Ringold, J. Med. Chem., 6 (1963) 195.
295. N.J. Doorenbos and C.P. Dorn, Jr., J. Pharm. Sci., 51 (1962) 414.
296. P.G. Holton and E. Necoechea, J. Med. Pharm. Chem., 5 (1962) 1352.

297. G. Nathansohn, E. Testa and N. Di Mola, Experientia, 18 (1962) 57.
298. N.J. Doorenbos and C.P. Dorn, Jr., J. Pharm. Sci., 54 (1965) 1219.
299. G. Ohta, T. Takegoshi, T. Onodera, A. Kasahara, Y. Oshima, M. Shimizu and K. Ueno, S. Afr. Patent 641,540 (1964).
300. M. Shimizu, G. Ohta, K. Ueno, T. Takegoshi, Y. Oshima, A. Kasahara, T. Onodera, M. Mogi and H. Tachizawa, Chem. Pharm. Bull., 13 (1965) 895.
301. G. Ohta, T. Takegoshi, K. Ueno and M. Shimizu, Chem. Pharm. Bull., 13 (1965) 1445.
302. R.E. Havranek, G.B. Hoey and D.H. Baeder, J. Med. Chem., 9 (1966) 326.
303. A. Kasahara, T. Onodera, M. Mogi, Y. Oshima and M. Shimizu, Chem. Pharm. Bull., 13 (1965) 1460.
304. A. Kasahara, T. Onodera, H. Tachizawa, Y. Oshima and M. Shimizu, Chem. Pharm. Bull., 14 (1966) 285.
305. T. Takegoshi, H. Tachizawa and G. Ohta, Chem. Pharm. Bull., 20 (1972) 1243.
306. T. Takegoshi, Chem. Pharm. Bull., 20 (1972) 1260.
307. K. Ueno and G. Ohta, Chem. Pharm. Bull., 15 (1967) 518.
308. A. Kasahara, T. Onodera, Y. Oshima and M. Shimizu, Chem. Pharm. Bull., 16 (1968) 1456.
309. A. Kasahara, Chem. Pharm. Bull., 16 (1968) 1460.
310. C.W. Howard, S.G. Hanson and M.A. Wahed, Br. Med. J., 1 (1977) 1659.
311. C.A. Forteza, A.J. Monasterio, P.M. Pico and C.A. Ribera, Med. Clin., 63 (1974) 383.
312. T. Kumada and Y. Abiko, Thromb. Haemostas., 36 (1976) 451.
313. T. Colton and I. Loas, U.S. Patent 2,999,092 (1961); Chem. Abstr., 56 (1962) 484.
314. P. de Ruggieri, C. Gandolfi and D. Chiaramonti, Gazz. Chim. Ital., 92 (1962) 768.
315. J.H. Ackerman, G.O. Potts, A.L. Beyler and R.O. Clinton, J. Med. Chem., 7 (1964) 238.
316. P. de Ruggieri and C. Gandolfi, Hormonal Steroids, Biochemistry Pharmacology and Therapeutics: Proc. 1st Int. Congr. Hormonal Steroids (Academic Press, New York, 1965) Vol. II, p. 69.
317. L.L. Smith, D.M. Teller and T. Foell, J. Med. Chem., 6 (1963) 330.
318. P.E. Shaw, F.W. Gubitz, K.F. Jennings, G.O. Potts, A.L. Beyler and R.L. Clarke, J. Med. Chem., 7 (1964) 555.
319. M. Shimizu, G. Ohta, K. Ueno and T. Takegoshi, Chem. Pharm. Bull., 12 (1964) 77.
320. N.J. Doorenbos and M.T. Wu, J. Med. Chem., 11 (1968) 158.
321. T.C. Miller and R.G. Christiansen, J. Org. Chem., 29 (1964) 3612.
322. M.E. Wolff, W. Ho and R. Kwok, J. Med. Chem., 7 (1964) 577.
323. P.D. Klimstra, E.F. Nutting and R.E. Counsell, J. Med. Chem., 9 (1966) 693.
324. K. Takeda, T. Komeno, J. Kawanami, S. Ishihara, H. Kadohawa, H. Takura and H. Itani, Tetrahedron, 21 (1965) 329.
325. T. Miyake and A. Tanaka, Ann. Rep. Shionogi Res. Lab., 19 (1969) 20.
326. T. Miyake, F. Kobayashi and T. Hori, Ann. Rep. Shionogi Res. Lab., 20 (1970) 55.
327. T. Komeno, S. Hayashi, S. Ishihara, H. Itani, H. Iwakura and K. Takeda, Ann. Rep. Shionogi Res. Lab., 19 (1969) 3.
328. T. Miyake, T. Komeno and K. Takeda, Ann. Rep. Shionogi Res. Lab., 19 (1969) 39.
329. T. Miyake, K. Uchida, H. Kakushi, Y. Nomura, M. Kadowaki, K. Miyata, T. Hanafusa and R. Muranaka, Japan. J. Pharmacol., 24 (1974) 551.
330. T. Miyake, K. Uchida, H. Kakushi, T. Hanafusa and R. Muranaka, Ann. Rep. Shionogi Res. Lab., 24 (1974) 51.
331. T. Miyake, K. Uchida, Y. Nomura, M. Kadowaki and K. Miyata, Ann. Rep. Shionogi Res. Lab., 24 (1974) 57.

332. K. Uchida, Y. Nomura, M. Kadowaki, K. Miyata and T. Miyake, Ann. Rep. Shionogi Res. Lab., 24 (1974) 55.
333. A. Tanaka, M. Nakamura, K. Odaguchi, T. Furukawa, T. Kawabata, K. Nakayama and T. Shike, Ann. Rep. Shionogi Res. Lab., 24 (1974) 84.
334. E. Farkas and J.M. Owen, J. Med. Chem., 9 (1966) 510.
335. H.G. Lehmann, O. Engelfried and R. Wiechert, J. Med. Chem., 8 (1965) 383.
336. R.F.R. Church, A.S. Kende and M.J. Weiss, J. Am. Chem. Soc., 87 (1965) 2665.
337. P. de Ruggieri, C. Gandolfi and D. Chiaramonti, Boll. Soc. Ital. Biol. Sper., 38 (1962) 985.
338. P. de Ruggieri, R. Matscher, C. Gandolfi, D. Chiaramonti, C. Lupo, E. Pietra and R. Cavalli, Arch. Sci. Biol., 47 (1963) 1.
339. R. Matscher, C. Lupo and P. de Ruggieri, Boll. Soc. Ital. Biol. Sper., 38 (1962) 988.
340. R.H. Wiley and S.H. Chang, J. Med. Chem., 6 (1963) 610.
341. C.H. Gleason, J. Med. Chem., 7 (1964) 573.
342. R.T. Blickenstaff, J. Med. Chem., 11 (1968) 1106.
343. G.R. Pettit, R.L. Smith and H. Klinger, J. Med. Chem., 10 (1967) 145.
344. R.M. Scribner, J. Org. Chem., 30 (1965) 3203.
345. D. Lednicer and J.C. Babcock, J. Org. Chem., 27 (1962) 2541.
346. R. Gaunt, J.H. Leatham, C.H. Tuthill, N. Antonchak, M. Gilman and A.A. Renzi, Endocrinology, 54 (1954) 272.
347. A.D. Cross, I.T. Harrison, P. Crabbé, F.A. Kincl and R.I. Dorfman, Steroids, 4 (1964) 229.
348. A.D. Cross and I.T. Harrison, Steroids, 6 (1965) 397.
349. E. Chang and V.K. Jain, J. Med. Chem., 9 (1966) 433.
350. R.I. Dorfman and F.A. Kincl, Endocrinology, 72 (1963) 259.
351. R.I. Dorfman and F.A. Kincl, Steroids, 1 (1963) 185.
352. F.A. Kincl and R.I. Dorfman, Steroids, 3 (1964) 109.
353. J.C. Orr, A. de la Roz and A. Bowers, J. Org. Chem., 29 (1964) 3300.
354. M.E. Wolff and T. Jen, J. Med. Chem., 6 (1963) 726.
355. M.E. Wolff, W. Ho and R. Kwok, Steroids, 5 (1965) 1.
356. M.E. Wolff and R.C. Boguslaski, J. Med. Chem., 11 (1968) 285.
357. B. Camerino, B. Patelli and A. Vercellone, J. Am. Chem. Soc., 78 (1956) 3540.
358. G. Sala and G. Baldratti, Proc. Soc. Exp. Biol. Med., 95 (1957) 22.
359. G. Sala, G. Baldratti and R. Rouchi, Folia Endocrinol., 10 (1957) 729.
360. A. Boris and M. Uskoković, Experientia, 26 (1970) 9.
361. A. Boris, L. DeMartino and T. Trmal, Endocrinology, 88 (1971) 1086.
362. A. Segaloff and R.B. Gabbard, Steroids, 4 (1964) 433.
363. F.R. Mangan and W.I.P. Mainwaring, Steroids, 20 (1972) 331.
364. L.J. Lerner, A.V. Bianchi and M. Dzelzkalns, Steroids, 6 (1965) 223.
365. L.J. Lerner, A. Bianchi and A. Borman, Cancer, 13 (1960) 1201.
366. D.M. Piatak, R.I. Dorfman, D. Tibbetts and E. Caspi, J. Med. Chem., 7 (1964) 590.
367. N.J. Doorenbos and C.R. Tamorria, J. Pharm. Sci., 54 (1965) 1473.
368. S.K. Saksena and R.R. Chaudhury, Indian J. Med. Res., 58 (1970) 513.
369. S.K. Saksena and R.R. Chaudhury, J. Reprod. Fert., 21 (1970) 559.
370. P. Kurath and W. Cole, J. Org. Chem., 26 (1961) 4592.
371. A. Kasal and O. Linet, Coll. Czech. Chem. Commun., 34 (1969) 3479.
372. A.I. Terekhina, G.I. Gritsina, A.V. Kamernitskii, L.I. Lisitsa, N.N. Neshchadim, N.S. Pavlova-Grishina and A.V. Skonva, Khim-Farm. Zh., 10 (1976) 31.

373. T.J. Bardos, Z.F. Chmielewicz, S.P. Raman and A. Segaloff, Steroids, 2 (1963) 105.
374. S.P. Raman, Z.F. Chmielewicz and T.J. Bardos, J. Med. Chem., 7 (1964) 678.
375. R.F. Spark and J.C. Melby, Ann. Int. Med., 69 (1968) 685.
376. R. Caminos-Torres, L. Ma and P.J. Synder, J. Clin. Endocrinol. Metab., 45 (1977) 255.
377. J.E. Castro, H.J. Griffiths and E.D. Edwards, Br. J. Surg., 58 (1971) 485.
378. P.D. Broulik and L. Starka, Endokrinologie, 68 (1976) 35.
379. S.L. Steelman, J.R. Brooks, E.R. Morgan and D.J. Patanelli, Steroids, 14 (1969) 449.
380. G.T. Basinger and R.F. Gittes, J. Urol., 111 (1974) 77.
381. P.J. Pentikäinen, L.A. Pentikäinen, D.H. Huffman and D.L. Azarnoff, J. Int. Med. Res., 2 (1974) 439.
382. J.A. Mahoudean, P. Corvol, J.C. Valcke, J. Menard and H. Bricaire, Ann. Endocrinol., 37 (1976) 57.
383. H.C. Erbler, Naunyn-Schmiedeberg's Arch. Pharmacol., 285 (1974) 403.
384. J.F. Dymling, K.O. Nilsson and B. Hökfelt, Acta Endocrinol., 70 (1972) 104.
385. P. Corvol, J.A. Mahoudean, J.C. Valcke, J. Menard and H. Bricaire, Nouv. Presse Med., 5 (1976) 691.
386. R.J. Santen, H.E. Kulin, D.L. Loriaux and J. Friend, J. Clin. Endocrinol. Metab., 43 (1976) 1386.
387. J.C. Pita Jr., M.E. Lippman, E.B. Thompson and D.L. Loriaux, Endocrinology, 97 (1975) 1521.
388. D.L. Loriaux, R. Menard, A. Taylor, J.C. Pita and R. Santen, Ann. Int. Med., 85 (1976) 630.
389. G.H. Rasmusson, A. Chen, G.F. Reynolds, D.J. Patanelli, A.A. Patchett and G.E. Arth, J. Med. Chem., 15 (1972) 1165.
390. Anonymous, Lancet, 1 (1976) 1003.
391. R. Wiechert, H. Steinbeck, W. Elger and F. Neumann, Arzneim. Forsch., 17 (1967) 1103.
392. R. Wiechert, Experientia, 24 (1968) 767.
393. A.S. Bhargava, A. Seeger and P. Günzel, Steroids, 30 (1977) 407.
394. E.L. Shapiro, L. Weber, H. Harris, C. Miscowicz, R. Neri and H.L. Herzog, J. Med. Chem., 15 (1972) 716.
395. D.M. Brennan and R.J. Kraay, Acta Endocrinol., 44 (1963) 367.
396. S. Honma, S. Iwamura, K. Iizuka, A. Kambegawa and K. Shida, Chem. Pharm. Bull., 25 (1977) 2019.
397. Y. Suzuki, Jap. Patent 17,673 (1963); Chem. Abstr., 60 (1964) 599.
398. A. Segaloff and R.B. Gabbard, Steroids, 5 (1965) 219.
399. F.J. Saunders, E.F. Nutting, R.E. Counsell and P.D. Klimstra, Proc. Soc. Exp. Biol. Med., 113 (1963) 637.
400. G.E. Arth, A.A. Patchett, T. Jefopoulus, R.L. Bugianesi, L.H. Peterson, E.A. Ham, F.A. Kuehl, Jr. and N.G. Brink, J. Med. Chem., 14 (1971) 675.
400a. R.J. Begue, J.-A. Gustafsson and A.S. Goldman, Endocrinology, 95 (1974) 238.
401. C.L. Rassaert, G. DiPasquali, T. Giannina, J.P. Manning and A. Meli, Steroids Lipids Res., 4 (1973) 333.
402. A. Boris, Steroids, 11 (1968) 681.
403. P. Kurath and R. Oslapas, J. Med. Chem., 12 (1969) 5.
404. A. Dinner, R.H. Bishara and B.S. Rutherford, J. Med. Chem., 18 (1975) 314.
405. J.W. Dean, G.O. Potts and R.G. Christiansen, J. Med. Chem., 10 (1967) 795.
406. C. Revesz and Y. Lefebvre, J. Med. Chem., 18 (1975) 217.

407. C. Revesz, U.K. Banik and Y. Lefebvre, J. Reprod. Fert., 22 (1970) 27.
408. Y. Lefebvre and C. Revesz, J. Med. Chem., 18 (1975) 581.
409. H. Laurent and G. Schulz, Chem. Ber., 102 (1969) 3324.
410. Y. Lefebvre, D.J. Marshall and C. Revesz, J. Med. Chem., 18 (1975) 220.
411. K. Ponsold, M. Hübner, R. Schnabel and J. Strecke, Arzneim. Forsch., 24 (1974) 896.
412. C. Hörhold, M. Hübner, K. Ponsold, R. Schnabel and K. Schubert, Pharmazie, 30 (1975) 35.
413. K. Rudolf and G. Goeretzlehner, Zbl. Gynak., 97 (1975) 108.
414. T.L. Patton and A. Dmochowski, Arch. Biochem. Biophys., 101 (1963) 181.
415. R.J. Fisette and L.R. Morgan, Arch. Int. Pharmacodyn. Ther., 168 (1967) 312.
416. D.G. Mikolasak, D.G. Gallo, J.L. Minielli, G.R. McKinney and A.A. Larsen, J. Med. Chem., 12 (1969) 1105.
417. A.D. Cross, E. Denot, H. Carpio, R. Acevedo and P. Crabbé, Steroids, 5 (1965) 557.
418. G. Baldratti, W. Barbieri, A. Consonni, R. Sciaky, E. Scrascia and G.K. Suchowsky, Experientia, 25 (1969) 1018.
419. T. Miyake and K. Takeda, Excerpta Medica Found. Int. Congr. Ser. No. 132 (1967) 616.
420. T. Miyake, T. Hori and T. Suzuki, Endocrinol. Japon., 20 (1973) 157.
421. T. Miyake, T. Hori, G. Kato, M. Ide, N. Uchida and K. Yamaguchi, Steroids, 23 (1974) 929.
422. E.A. Brown, J. Med. Chem., 10 (1967) 546.
423. J.R. Brooks, S.L. Steelman and D.J. Patanelli, Steroids, 29 (1977) 809.
424. G. Bialy, A.P. Merril and G. Pincus, Endocrinology, 79 (1966) 125.
425. B. Singh and R.G. Christiansen, J. Pharm. Sci., 60 (1971) 491.
426. C.W. Emmens, J. Reprod. Fertil., 9 (1965) 277.
427. I. Pollard and L. Martin, Steroids, 11 (1968) 897.
428. E.R. Clark, A.M.E. Omar and G. Prestwich, J. Med. Chem., 20 (1977) 1096.
429. A.P. Shroff, J. Med. Chem., 13 (1970) 748.
430. Ch. R. Engel, S. Salvi and M.N. Roy Chowdhury, Steroids, 25 (1975) 781.
431. R.A. LeMahieu, A. Boris, M. Carson, R.W. Guthrie and R.W. Kierstead, J. Med. Chem., 16 (1973) 647.
432. D. Bertin and J. Perronnet, Bull. Soc. Chim. Fr. (1969) 117.
433. R.E. Brown, D.M. Lustgarten, R.J. Stanaback and R.I. Meltzer, J. Med. Chem., 10 (1967) 451.
434. J. Bowler, and R. Clarkson, J. Chem. Soc. (C), (1968) 2975.
435. M.E. Wolff and G. Zanati, J. Med. Chem., 13 (1970) 563.
436. P.D. Klimstra, J. Med. Chem., 9 (1966) 781.
437. D. van der Sijde, H.J. Kooreman, K.D. Jaitley and A.F. Marx, J. Med. Chem., 15 (1972) 909.
438. A.F. Marx, H.J. Kooreman, K.D. Jaitley and D. van der Sijde, J. Med. Chem., 16 (1973) 1302.
439. A.P. Shroff, J. Med. Chem., 8 (1965) 881.
440. M.E. Wolff and C. Hansch, J. Med. Chem., 17 (1974) 898.
441. J.G. Topliss and E.L. Shapiro, J. Med. Chem., 18 (1975) 621.
442. H. Laurent, G. Schulz and R. Wiechert, Chem. Ber., 102 (1969) 2570.
443. A.P. Shroff, R.P. Blye and J.L. McGuire, J. Med. Chem., 14 (1971) 769.
444. M.J. Weiss, J.F. Poletto, G.R. Allen, Jr., R.E. Schaub and I. Ringler, J. Med. Chem., 7 (1964) 804.

445. K.E. Fahrenholtz, A. Boris, T.W. Kennedy, Jr. and R.W. Kierstead, J. Med. Chem., 17 (1974) 337.
446. B. Beyer, L. Terenius, R.W. Brueggemeier, V.V. Ranade and R.E. Counsell, Steroids, 27 (1976) 123.
447. A.P. Shroff, C.H. Harper, G.O. Allen and R.P. Blye, J. Med. Chem., 16 (1973) 113.
448. D.W. Hahn, G.O. Allen and J.L. McGuire, Contraception, 16 (1977) 541.
449. S.F. Sisenwine, A.L. Liu, H.B. Kimmel and H.W. Ruelius, Contraception, 15 (1977) 25.
450. J.E. Patrick, K.B. Alton, N. Hetyei and C. Shaw, Pharmacologist, 18 (1976) 153.
451. H.G. McQuarrie, J.W. Harris, S.A. Pasquale and P.J. Santella, Obstet. Gynecol. Digest, (1972) 21.
452. J. Kärkhainen, J.J. Ohisalo and T. Luuhkainen, Contraception, 12 (1975) 511.
453. A.F. Hirsch, G.O. Allen, B. Wong, S. Reynolds, C. Exarhos, W. Brown and D.W. Hahn, J. Med. Chem., 20 (1977) 1546.
454. U.K. Banik, C. Revesz and F. Herr, J. Reprod. Fertil., 18 (1969) 509.
455. S.K. Saksena and R.R. Chaudhury, J. Reprod. Fertil., 19 (1969) 177.
456. W.P. Dmowski, H.F.L. Scholer, V.B. Mahesh and R.B. Greenblatt, Fertil. Steril., 22 (1971) 9.
457. G.O. Potts, A.L. Beyler and H.P. Schane, Fertil. Steril., 25 (1974) 367.
458. R.B. Greenblatt, W.P. Dmowski, V.B. Mahesh and H.F.L. Scholer, Fertil. Steril., 22 (1971) 102.
459. R.L. Friedlander, J. Reprod. Med., 10 (1973) 197.
460. R.J. Sherins, H.M. Gandy, T.W. Thoruslund and C.A. Paulsen, J. Clin. Endocrinol., 32 (1971) 522.
461. R.L. Barbieri, J.A. Canick, A. Makris, R.B. Todd, I.J. Davies and K.J. Ryan, Fertil. Steril., 28 (1977) 809.
462. W.P. Dmowski and M.R. Cohen, Obstet. Gynecol., 46 (1975) 147.
463. G.P. Wood, C.-H. Wu, G.L. Flickinger and G. Mikhail, Obstet. Gynecol., 45 (1975) 302.
464. N.H. Lauersen, K.H. Wilson and S. Birnbaum, Am. J. Obstet. Gynecol., 123 (1975) 742.
465. T. Lind and D.B. Cook, Lancet, 1 (1976) 1401.
466. H. Jacobs, J. Reprod. Med., 19 (1977) 313.
467. D.H. Hussar, Am. J. Pharm., 149 (1977) 5.
468. R.H. Asch and R.B. Greenblatt, Am. J. Obstet. Gynecol., 129 (1977) 130.
469. H.P. Schane and G.O. Potts, Fertil. Steril., 25 (1974) 363.
470. R.B. Greenblatt, R. Borenstein, C.S.S. Bohler, J. Reprod. Med., 13 (1974) 201.
471. M.L. Colle and R.B. Greenblatt, J. Reprod. Med., 17 (1976) 98.
472. N.H. Lauersen and K.H. Wilson, Obstet. Gynecol., 50 (1977) 91.
473. C. Davison, W. Banks and A. Fritz, Arch. Int. Pharmacodyn. Ther., 221 (1976) 294.
474. D. Rosi, H.C. Neumann, R.G. Christiansen, H.P. Schane and G.O. Potts, J. Med. Chem., 20 (1977) 349.
475. G.O. Potts, U.S. Patent 3,966,926 (1976); Chem. Abstr., 85 (1976) 83244.
476. J.E. Wooford and W.H. Elder, J. Wildlife Management, 31 (1967) 507.
477. R.E. Counsell, M.C. Lu, S. El Masry and P.A. Weinhold, Biochem. Pharmacol., 20 (1971) 2912.
478. R.D. Skoglund and A.P. Paulsen, Contraception, 7 (1973) 357.
479. M. Ulstein, N. Netto, J. Leonard, and C.A. Paulsen, Contraception, 12 (1975) 437.

480. U.J. Koch, F. Lorenz, K. Danehl, R. Ericsson, S.H. Hasan, D.V. Keyserlingk, K. Lülke, M. Mehring, A. Römmler, U. Schwartz and J. Hammerstein, Contraception, 14 (1976) 117.
481. S. Roy, S. Chatterjee, M.R.N. Prasad, A.K. Poddar, D.C. Pandey, H.C. Pandey and Y.N. Jadhau, Contraception, 14 (1976) 403.
482. B.H. Shapiro and A.S. Goldman, Proc. Soc. Exp. Biol. Med., 150 (1975) 226.
483. A.S. Goldman, B.H. Shapiro and A.W. Root, J. Endocrinol., 69 (1976) 11.
483a. M.S. Manhas, B. Lal and A.K. Bose, 169th Nat. Meeting Am. Chem. Soc., Philadelphia, (1975) Abstr. no. 47.
484. H. Selye, Rev. Can. Biol., 29 (1970) 49.
485. H. Selye, J. Pharm. Sci., 60 (1971) 1.
486. P. Kourounakis, Am. J. Pharm., 146 (1974) 22.
487. B. Solymoss, S. Tóth, S. Varga and H. Selye, Toxicol. Appl. Pharmacol., 18 (1971) 586.
488. J. Romo, Tetrahedron, 3 (1958) 37.
489. B. Ellis, V. Petrow and D. Wedlake, J. Chem. Soc., Part 3 (1958) 3748.
490. H. Selye in: Hormone and Resistance (Springer-Verlag, Heidelberg, 1970).
491. A. Somogyi, K. Kovacs, B. Solymoss, R. Kuntzman and A.H. Conney, Pharmacologist, 13 (1971) 244.
492. P. Kourounakis, Pharmacologist, 16 (1974) 195.
493. J. Taché, Y. Taché, P. Kourounakis and H. Selye, Biochem. Exp. Biol., 11 (1975) 351.
494. P. Kourounakis, Biochem. Exp. Biol., 12 (1976) 317.
495. A. Terzis, P. Kourounakis and T. Theopanides, J. Pharm. Sci., 65 (1976) 1092.
496. S. Brookman and P. Kourounakis, J. Pharm. Sci., 66 (1977) 1492.
497. Y. Taché, J. Taché and H. Selye, Arzneim. Forsch., 25 (1975) 1603.
498. B.D. Garg, K. Kovacs, J.A. Blascheck and H. Selye, Folia Endocrinol., 23 (1970) 357.
499. B. Solymoss, J. Werringloer and S. Tóth, Steroids, 17 (1971) 427.
500. P. Kourounakis, S. Szabo, J. Werringloer and H. Selye, J. Pharm. Sci., 62 (1973) 690.
501. S. Szabo, P. Kourounakis, H. Selye and O. DaSilva, J. Pharmacol. Exp. Ther., 188 (1974) 45.
502. B.D. Garg, S. Szabo, B. Tuchweber and K. Kovacs, Acta Anat., 90 (1974) 87.
503. B.D. Garg, K. Kovacs, B. Tuchweber and J.D. Khandekar, Acta Anat., 91 (1975) 161.
504. S. Szabo, H. Selye, I. Japundzic, J. Mimic-oka and M. Japundzic, Fed. Proc., 31 (1972) 271.
505. A. Somogyi, K. Kovacs, B. Solymoss, R. Kuntzman and A.H. Conney, Life Sci. (II), 10 (1971) 1261.
506. K. Einarsson and J.Å. Gustafsson, Eur. J. Biochem., 32 (1973) 197.
507. R.E. Talcott and S.J. Stohs, Res. Commun. Chem. Pathol. Pharmacol., 5 (1973) 663.
508. A.H. Conney, A.Y.H. Lu, W. Levin, A. Somogyi, S. West, M. Jacobson, D. Ryan and R. Kuntzman, Drug Metab. Dispos., 1 (1973) 199.
509. P. Kourounakis, S. Szabo and H. Selye, Arzneim. Forsch., 26 (1976) 74.
510. I. Japundžić, M. Japundžić and S. Szabo, Eur. J. Biochem., 43 (1974) 79.
511. J. Werringloer, Physiologist, 14 (1971) 3.
512. J. Stohs and H. Rosenberg, J. Pharm. Sci., 65 (1976) 610.
513. L.S. Birnbaum, M.B. Baird and H.R. Massie, Res. Commun. Chem. Pathol. Pharmacol., 15 (1976) 553.
514. K. Von Bergmann, H.P. Schwarz and G. Paumgartner, Naunyn-Schmiedeberg's Arch. Pharmacol., 287 (1975) 33.

515. K. Einarsson and J.Å. Gustafsson, Biochem. Pharmacol., 23 (1974) 9.
516. H. Selye, Proc. Soc. Exp. Biol. Med., 141 (1972) 555.
517. Y. Taché, H. Selye and J. Taché, Endocrinology, 97 (1975) 731.
518. S. Szabo, S. Komlos and Z. Ignjatovic, J. Pharm. Pharmacol., 27 (1975) 113.
519. H. Selye, Steroids, 13 (1969) 803.
520. K. Kovacs and A. Somogyi, Proc. Soc. Exp. Biol. Med., 131 (1969) 1350.
521. C.P. Robinson, P.W. Smith, J.K. McConnell and B.R. Endecott, J. Pharm. Sci., 65 (1976) 595.
522. C.D. Klaassen, Toxicol. Appl. Pharmacol., 38 (1976) 127.
523. D.A. Haugen, M.J. Coon and D.W. Nebert, J. Biol. Chem., 251 (1976) 1817.
524. H. Selye, M. Krajny and L. Savoie, Science, 164 (1969) 842.
525. H. Selye, J. Mécs and T. Tamura, Br. J. Pharmacol., 37 (1969) 485.
526. H. Selye, J. Jelinek and M. Krajny, J. Pharm. Sci., 58 (1969) 1055.
527. S.H. Buck and G.L. Lage, Arch. Int. Pharmacodyn. Ther., 189 (1971) 192.
528. M.C. Castle and G.L. Lage, Biochem. Pharmacol., 21 (1972) 1449.
529. M.C. Castle and G.L. Lage, Drug Metab. Dispos., 1 (1973) 590.
530. M.C. Castle and G.L. Lage, Toxicol. Appl. Pharmacol., 27 (1974) 641.
531. K.E. Wirth and J.C. Frölich, Eur. J. Pharmacol., 29 (1974) 43.
532. B. Stripp, M.E. Hamrick, N.G. Zampaglione and J.R. Gillette, J. Pharmacol. Exp. Ther., 176 (1971) 766.
533. K.E. Wirth, J.C. Frölich, J.W. Hollifield, F.C. Falkner, B.S. Sweetman and J.A. Oates, Eur. J. Clin. Pharmacol., 9 (1976) 345.
534. G. Zsigmond and B. Solymoss, J. Pharmacol. Exp. Ther., 183 (1972) 499.
535. G. Zsigmond and B. Solymoss, Proc. Soc. Exp. Biol. Med., 145 (1974) 631.
536. F.M. Radzialowski, Biochem. Pharmacol., 22 (1973) 1607.
537. U. Abshagen, Naunyn-Schmiedeberg's Arch. Pharmacol., 278 (1973) 91.
538. U. Abshagen, H. Rennekamp and J. Kuhlmann, Naunyn-Schmiedeberg's Arch. Pharmacol., 292 (1976) 87.
539. K.-D. Krämer, P. Ghabussi and H. Hochrein, Arzneim. Forsch., 23 (1973) 508.
540. K.-D. Krämer, W. Vogt, P. Ghabussi and H. Hochrein, Med. Welt, 24 (1973) 462.
541. G.E. Musgrane, C.K. Born, C.P. Davidson and M.E. Hamrick, J. Pharmacol. Exp. Ther., 202 (1977) 696.
542. B. Solymoss, H.G. Classen and S. Varga, Proc. Soc. Exp. Biol. Med., 132 (1969) 940.
543. B. Solymoss, S. Varga and H.G. Classen, Eur. J. Pharmacol., 10 (1970) 127.
544. D.R. Feller and M.C. Gerald, Biochem. Pharmacol., 20 (1971) 1991.
545. S.A. Taylor, M.D. Rawlins and S.E. Smith, J. Pharm. Pharmacol., 24 (1972) 578.
546. D.H. Huffman, D.W. Shoeman, P. Pentikäinen and D.L. Azarnoff, Pharmacology, 10 (1973) 338.
547. M.E. Hamrick, N.G. Zampaglione, B. Stripp and J.R. Gillette, Biochem. Pharmacol., 22 (1973) 293.
548. B. Solymoss, M. Krajny, S. Varga and J. Werringloer, J. Pharmacol. Exp. Ther., 174 (1970) 473.
549. I.D. Mansurova, V.G. Chernyshov and R.Z. Stosman, Bull. Exp. Biol. Med., 76 (1974) 1049.
550. H. Selye, Perspect. Biol. Med., 13 (1970) 343.
551. K. Lehotzky, Int. Arch. Arbeitsmed., 33 (1974) 329.
552. M.C. Kushlan, J.E. Haddow and R. Lester, Toxicol. Appl. Pharmacol., 31 (1975) 527.
553. C.D. Klaasen, Toxicol. Appl. Pharmacol., 33 (1975) 366.

554. J.E. Haddow, C.A. Fish, P.C. Marshall and R. Lester, Gastroenterology, 63 (1972) 1053.
555. M. Cikrt and M. Tichy, Environ. Res., 10 (1975) 427.
556. J.E. Haddow and R. Lester, Drug Metab. Dispos., 4 (1976) 499.
557. K. Kitani, Y. Morita and S. Kanai, Biochem. Pharmacol., 26 (1977) 279.
558. B.D. Garg, B. Solymoss and B. Tuchweber, Arzneim. Forsch., 21 (1971) 815.
559. K. Kitani, R. Miura, S. Kanai and Y. Morita, Biochem. Pharmacol., 26 (1977) 1823.
560. K. Bjondahl, M. Möttönen and L. Nieminen, Experientia, 29 (1973) 685.
561. K. Bjondahl, B. Isomaa and L. Nieminen, Biochem. Pharmacol., 23 (1974) 1509.
562. R. Thomas, J. Boutagy and A. Gelbart, J. Pharm. Sci., 63 (1974) 1649.
563. A. Schwartz, G.E. Lindenmayer and J.C. Allen, Pharmacol. Rev., 27 (1975) 3.
564. C.L. Stevens, G.H. Ransford and G.E. Gutowski, Am. Chem. Soc. Abstr. Med. (1968) 109.
565. M.M. Janot, M. Leboeuf, A. Cave, R. Wijesekera and R. Goutarel, C.R. Acad. Sci. (Paris), Ser. C, 267 (1968) 1050.
566. R.W. Caldwell and C.B. Nash, J. Pharmacol. Exp. Ther., 197 (1976) 19.
567. L.S. Cook, R.W. Caldwell and C.B. Nash, Pharmacologist, 18 (1976) 123.
568. L.S. Cook, R.W. Caldwell and C.B. Nash, Arch. Int. Pharmacodyn., 227 (1977) 220.
569. O. Foussard-Blanpin, F. Hubert, P. Choay and M. Leboeuf, Ann. Pharm. Fr., 31 (1973) 593.
570. T. Akera, S.I. Baskin, T. Tobin and T.M. Brody, Naunyn-Schmiedeberg's Arch. Pharmacol., 277 (1973) 151.
571. L. Hokin, M. Mokotoff and S.M. Kupchan, Proc. Nat. Acad. Sci., 55 (1966) 797.
572. T. Tobin and H. Abramson, Eur. J. Pharmacol., 32 (1975) 243.
573. T. Tobin, T. Akera, D. Ku and M.C. Lu, Mol. Pharmacol., 9 (1973) 676.
574. T. Tobin, T. Akera, T.M. Brody and H.R. Taneja, Eur. J. Pharmacol., 35 (1976) 69.
575. Y. Saito, Y. Kanemasa and M. Okada, Chem. Pharm. Bull., 18 (1970) 629.
576. K. Takeda, T. Shigei and S. Imai, Experientia, 26 (1970) 867.
577. W. Zürcher, E. Weiss-berg and C. Tamm, Helv. Chim. Acta, 52 (1969) 449.
578. T.R. Witty, W.A. Remers and H.R. Besch, Jr., J. Pharm. Sci., 64 (1975) 1248.
579. L. Sawlewicz, E. Weiss, H.H.A. Linde and K. Meyer, Helv. Chim. Acta, 55 (1972) 2452.
580. H.N. Abramson, C.L. Huang, T.F. Wu and T. Tobin, J. Pharm. Sci., 65 (1976) 765.
581. H.N. Abramson, C.L. Huang, C.-C. Ho, L.L. Walny and Y.-M. Hsu, J. Pharm. Sci., 66 (1977) 602.
582. M. Okada and T. Anjyo, Chem. Pharm. Bull., 25 (1977) 3090.
583. H. Tsuru, N. Ishikawa, T. Shigei, T. Anjyo and M. Okada, Japan. J. Pharmacol., 27 (1977) 799.
584. W. Eberlein, J. Nickl, J. Heider, G. Dahms and H. Machleidt, Chem. Ber., 105 (1972) 3686.
585. R. Megges, H. Timm, P. Thiemann, F. Dittrich, P. Franke, H. Portius and K. Repke, Ger. (East) Patent 109, 622 (1974); Chem. Abstr., 83 (1975) 59166.
586. R. Deghenghi, Pure Appl. Chem., 21 (1970) 153.
587. G. Pastelin and R. Mendez, Eur. J. Pharmacol., 19 (1972) 291.
588. R. Mendez, G. Pastelin and E. Kabela, J. Pharmacol. Exp. Ther., 188 (1974) 189.
589. J.I. Gliklich, R. Gaffney, M.R. Rosen and B.F. Hoffman, Eur. J. Pharmacol., 32 (1975) 1.
590. G.R. Pettit, B. Green and G.L. Dunn, J. Org. Chem., 35 (1970) 1377.
591. G.R. Pettit, B. Green, A.K. Das Gupta, P.A. Whitehouse and J.P. Yardley, J. Org. Chem., 35 (1970) 1381.

592. T. Minesita, R. Hirota, S. Kimoto, M. Uno and Y. Uemura, Ann. Rep. Shionogi Res. Lab., 18 (1968) 94.
593. S. Matsumura, S. Kimoto, O. Uno, T. Minesita and M. Ueda, Arch. Int. Pharmacodyn., 225 (1977) 246.
594. B.G. Katzung, J.A. Munoz, D.Y. Shirachi, A.J. Trevor, H.H. Chang and M.E. Wolff, Experientia, 26 (1970) 1189.
595. J.M. Ferland and Y. Lefebvre, U.S. Patent 3,462,413 (1969); Chem. Abstr., 71 (1969) 113228.
596. J.W. Ralls and C.G. Bergstrom, U.S. Patent 2,793,207 (1957); Chem. Abstr., 52 (1958) 464.
597. R.E. Schaub and M.J. Weiss, J. Org. Chem., 26 (1961) 1223.
598. K. Takamura, C. Isono, S. Takaku and Y. Nitta, Chem. Pharm. Bull., 11 (1963) 604.
599. K. Takamura, C. Isono, S. Takama and Y. Nitta, Chem. Pharm. Bull., 11 (1963) 613.
600. M.E. Wolff, H.-H. Chang and W. Ho, J. Med. Chem., 13 (1970) 657.
601. S. Goya, K. Shimada, J. Goto, S. Usuda and T. Nambara, Yakugaku Zasshi, 90 (1970) 537.
602. T. Nambara, K. Shimada, T. Nemoto and S. Goya, Chem. Pharm. Bull., 18 (1970) 1658.
603. T. Nambara, T. Shibata, M. Mimura and H. Hosoda, Chem. Pharm. Bull., 19 (1971) 954.
604. W.R. Biggerstaff, N. Sugisaka and R. Albers, J. Med. Chem., 12 (1969) 1.
605. R. Thomas, J. Boutagy and A. Gelbart, J. Pharmacol. Exp. Ther., 191 (1974) 219.
605a. A. Gelbart and R. Thomas, J. Med. Chem., 21 (1978) 284.
606. N.A. Beard, W. Rouse and A.R. Somerville, Br. J. Pharmacol., 54 (1975) 65.
607. G. Kroneberg, K.H. Meyer, E. Schraufstätter, S. Schütz and K. Stoepel, Naturwissenschaften, 51 (1964) 192.
608. G. Kroneberg and K. Stoepel, Arch. Exp. Pathol. Pharmakol., 249 (1964) 393.
609. S. Schütz, K. Meyer and H. Krätzer, Arzneim. Forsch., 19 (1969) 69.
610. B. Duhm, W. Maul, H. Medenwald, K. Patzschke and I. Wagner, Arzneim. Forsch., 19 (1969) 1978.
611. H.G. Kroneberg, Actual. Pharmacol., 25 (1972) 157.
612. R.E. Brown, D.M. Lustgarten, R.J. Stanaback, M.W. Osborne and R.I. Meltzer, J. Med. Chem., 7 (1964) 232.
613. H.D. Brown, Biochem. Pharmacol., 15 (1966) 2007.
614. M.W. Osborne and M.M. Winbury, J. Pharmacol. Exp. Ther., 147 (1965) 212.
615. M.W. Osborne, H.M. Rowe, R.N. Kaufman, M.C. Johnson, W. Willems and M.M. Winbury, J. Pharmacol. Exp. Ther., 147 (1965) 225.
616. B. Dubnick, D.F. Morgan, C.A. Towne and G.E. Phillips, J. Pharmacol. Exp. Ther., 153 (1966) 301.
617. D. Bertin, U.S. Patent 3,054,812 (1962); Chem. Abstr., 58 (1963) 9194.
618. D. Bertin, A. Locatelli, J. Mathieu, G. Muller and H. Fritel, U.S. Patent 3,052,675 (1962); Chem. Abstr., 58 (1963) 2489.
619. D. Bertin, L. Nedelec and A. Locatelli, Belg. Patent 612,142 (1962); Chem. Abstr., 58 (1963) 1517.
620. Roussel-UCLAF, Belg. Patent 615,766 (1962); Chem. Abstr., 58 (1963) 12641.
621. Roussel-UCLAF, Fr. Patent M1598 (1963); Chem. Abstr., 59 (1963) 2912.
622. Roussel-UCLAF, Fr. Patent M1635 (1963); Chem. Abstr., 58 (1963) 12644.
623. Roussel-UCLAF, Fr. Patent M2478 (1964); Chem. Abstr., 61 (1964) 8367.

624. Roussel-UCLAF, Belg. Patent 618,714 (1962); Chem. Abstr., 58 (1963) 12638.
625. J. Le Men, J. Provost, R. Tiberghien, P. Forgacs, H. Eyrand and M. Aurousseau, Chim. Ther., 5 (1970) 41.
626. P.H. Morgan and I.W. Mathison, J. Pharm. Sci., 65 (1976) 467.
627. P.H. Morgan and I.W. Mathison, J. Pharm. Sci., 65 (1976) 635.
628. B.B. Vargaftig, M.F. Sugrue, W.R. Buckett and H. van Riezen, J. Pharm. Pharmacol., 27 (1975) 697.
629. R.J. Marshall and J.R. Parratt, Br. J. Pharmacol., 55 (1975) 359.
630. R.J. Marshall and J.R. Parratt, Br. J. Pharmacol., 61 (1977) 315.
631. W.J. Remme, P.D. Verdouw and F. Hagemeÿer, Eur. J. Pharmacol., 36 (1976) 189.
632. P.D. Verdouw, W.J. Remme, J. Beaune, A.M. Rutteman and F. Hagemeijer, Arzneim. Forsch., 26 (1976) 1558.
633. L.A. Salako, E.M.V. Williams and J.H. Wittig, Br. J. Pharmacol., 57 (1976) 251.
634. K.A. Kane, Brit. J. Pharmacol., 61 (1977) 457P.
635. W.R. Buckett, F.A. Marwick and B.B. Vargaftig, Br. J. Pharmacol., 54 (1975) 3.
636. W.T. Comer and A.W. Gomoll in: Medicinal Chemistry, Part II, ed. A. Burger (Wiley-Interscience, New York, 1970) p. 1019.
637. R.L. Lucas, D.F. Dickel, R.L. Dziemian, M.J. Ceglowski, B.L. Hensle and H.B. MacPhillamy, J. Am. Chem. Soc., 82 (1960) 5688.
637a. F.C. Uhle, J. Am. Chem. Soc., 73 (1951) 883.
638. C.G. Bergstrom, J. Med. Chem., 10 (1967) 440.
639. J.C. Kim, E. Lee, C.C. Chang and N.J. Doorenbos, Taehan Hwahak Hoechi, 19 (1975) 174; Chem. Abstr., 83 (1975) 172571.
640. F.M. Sturtevant, Proc. Soc. Exp. Biol. Med., 97 (1958) 619.
641. D. Gould, E.L. Shapiro, E.B. Hershberg, J. Am. Chem. Soc., 76 (1954) 5567.
642. N.J. Doorenbos and M.T. Wu, J. Pharm. Sci., 54 (1965) 1290.
643. C.M. Kagawa and R. Pappo, Proc. Soc. Exp. Biol. Med., 109 (1962) 982.
644. A.F. de Nicola, J.T. Oliver and M.K. Birmingham, Endocrinology, 83 (1968) 141.
645. A.F. de Nicola, H. Trackov, M.K. Birmingham, J.D. Palmer and D.W. Ruddick, J. Endocrinol. Metab., 30 (1970) 402.
646. M.P. Li, T.H. Chan and M.K. Birmingham, J. Steroid Biochem., 7 (1976) 949.
647. P. de Ruggieri, C. Gandolfi and D. Chiaramonti, Gazz. Chim. Ital., 93 (1963) 269.
648. C.H. Robinson, N.F. Bruce and E.P. Oliveto, J. Med. Chem., 6 (1963) 793.
649. R.E. Schaub, J.H. van den Hende and M.J. Weiss, J. Org. Chem., 30 (1965) 2234.
650. T.L. Popper, O. Gnoj and F.E. Carlon, J. Med. Chem., 13 (1970) 564.
651. O. Linét, Arzneim. Forsch., 19 (1969) 1899.
652. W.S. Allen, H.M. Kissman, S. Mauer, I. Ringler and M.J. Weiss, J. Med. Pharm. Chem., 5 (1962) 133.
653. J. Romo, G. Rosenkranz and C. Djerassi, J. Am. Chem. Soc., 73 (1951) 4961.
654. G.C. Buzby, Jr., R.A. Edgren, J.A. Fisher, G.A. Hughes, R.C. Jones, K. Ledig, T.W. Pattison, R. Rees, H. Smith, L.L. Smith, D.M. Teller and G.R. Wendt., J. Med. Chem., 7 (1964) 755.
655. A.H. Goldkamp, J. Med. Chem., 13 (1970) 561.
656. L.J. Chinn and K.W. Salamon, J. Med. Chem., 20 (1977) 229.
657. G.P. Mueller, W.F. Johns, D.L. Cook and R.A. Edgren, J. Am. Chem. Soc., 80 (1958) 1769.
658. G.P. Mueller and W.F. Johns, J. Org. Chem., 26 (1961) 2403.
659. W.F. Johns, J. Med. Chem., 12 (1969) 885.

660. A. Sachs and L. Wolfman, Metabolism, 17 (1968) 400.
661. C.S. Glueck, Metabolism, 20 (1971) 691.
662. C.S. Glueck, D. Scheel, J. Fishback and D. Steiner, Lipids, 7 (1972) 110.
663. O. Faergemen and F. Damgaard-Pederson, Scand. J. Clin. Lab. Invest., 31 (1973) 27.
664. E.S. Mills, B.M. Kriz and A.L. Jones, Proc. Soc. Exp. Biol. Med., 144 (1973) 861.
665. D.L. Schmucker and A.L. Jones, J. Lipid Res., 16 (1975) 143.
666. D. Kritchevsky, S.A. Tepper and J.A. Story, Arzneim. Forsch., 26 (1976) 862.
667. K. Nakamura, A. Toyota, Y. Masuda and H. Nakamura, J. Atheroscler. Res., 7 (1967) 783.
668. T. Onodera, T. Hashizume and A. Kasahara, Chem. Pharm. Bull., 21 (1973) 21.
669. K. Uchida, Y. Nomura, M. Kadowaki, K. Miyata and T. Miyake, Ann. Rep. Shionogi Res. Lab., 24 (1974) 63.
670. W. Nagata, T. Sugasawa, M. Narisada, T. Okada, K. Sasakura, M. Murakami and Y. Hayase, Chem. Pharm. Bull., 14 (1966) 174.
671. S. Gordon, E.W. Cantrall, W.P. Cekleniak, H.J. Albers, R. Littell and S. Bernstein, Biochem. Biophys. Res. Commun., 6 (1961) 359.
672. E.W. Cantrall, R. Littell, S.M. Stolar, W.P. Cekleniak, H.J. Albers, S. Gordon and S. Bernstein, Steroids, 1 (1963) 173.
673. R.G. Gould and C.B. Taylor, Fed. Proc., 9 (1950) 179.
674. G.M. Tomkins, H. Sheppard and I.L. Chaikoff, J. Biol. Chem., 201 (1953) 137.
675. I.D. Frantz, Jr., H.S. Schneider and B.T. Hinkelman, J. Biol. Chem., 206 (1954) 465.
676. E.P. Madhava Bhattathiry and M.D. Siperstein, J. Clin. Invest., 42 (1963) 1613.
677. R.E. Counsell, P.D. Klimstra, R.E. Ranney and D.L. Cook, J. Med. Pharm. Chem., 5 (1962) 720.
678. R.E. Counsell, P.D. Klimstra and R.E. Ranney, J. Med. Pharm. Chem., 5 (1962) 1224.
679. M.J. Thompson, J. Dupont and W.E. Robbins, Steroids, 2 (1963) 99.
680. L. Smith and J. Dupont, Steroids, 9 (1967) 85.
681. J.M. Martt and C.R. Talbert, Circulation, 28 (1963) 763.
682. R.E. Counsell, P.D. Klimstra, L.N. Nysted and R.E. Ranney, J. Med. Chem., 8 (1965) 45.
683. A.D. Cross, E. Denot, R. Acevedo and P. Crabbé, Steroids, 5 (1965) 585.
684. K. Irmscher, J.M. Kraemer and H. Halpaap, Steroids, 7 (1966) 557.
685. P.D. Klimstra, R.E. Ranney and R.E. Counsell, J. Med. Chem., 9 (1966) 323.
686. V.V. Ranade, F. Kohen and R.E. Counsell, J. Med. Chem., 14 (1971) 38.
687. F. Kohen, V.V. Ranade and R.E. Counsell, J. Med. Chem., 15 (1972) 1129.
688. M.C. Lu, F. Kohen and R.E. Counsell, J. Med. Chem., 14 (1971) 136.
689. C. Plotka, R. Deraedt, J. Pottier, P. Zirinis and R. Jequier, Arch. Int. Pharmacodyn., 152 (1964) 319.
690. D.L. Venton, F. Kohen and R.E. Counsell, J. Med. Chem., 16 (1973) 571.
691. J.A. Svoboda, T.R. Wrenn, M.J. Thompson, J.R. Weyant, D.L. Wood and J. Bitman, Lipids, 12 (1977) 691.
692. H. Pinhas, A. Loiseau, A. Krikorian-Manonkian, D. Courmarcel, N.P. Buu-Hoi and P. Jacquignon, J. Med. Chem., 14 (1971) 1048.
693. M. Gut, Y. Letourneus, J.A. Story, S.A. Tepper and D. Kritchevsky, Experientia, 30 (1974) 1325.
694. D.T. Witiak, R.A. Parker, M.E. Dempsey and M.C. Ritter, J. Med. Chem., 14 (1971) 684.
695. L.N. Nysted, U.S. Patent 4,001,220 (1977); Chem. Abstr., 87 (1977) 53494.

696. S. Burstein, Y. Letourneux, H.L. Kimball and M. Gut, Steroids, 27 (1976) 361.
697. M.C. Lu, P. Afiatpour, C.B. Sullivan and R.E. Counsell, J. Med. Chem., 15 (1972) 1284.
698. N. Winer, J.M. Martt, J.E. Somers, L. Wolcott, H.E. Dale and T.W. Burns, J. Lab. Clin. Med., 66 (1965) 758.
699. T.W. Burns, H.E. Dale and P.L. Langley, Am. J. Physiol., 209 (1965) 1227.
700. N. Winer, D.M. Klachko, R.D. Baer, P.L. Langley and T.W. Burns, Science, 153 (1966) 312.
701. J.B. Peter and W. Fiehn, Science, 179 (1973) 910.
702. J.B. Peter, R.M. Andiman, R.L. Bowman and T. Nagamoto, Exp. Neurol., 41 (1973) 738.
703. J.B. Peter, S.H. Dromgoole, D.S. Campion, K.E. Stempel, R.L. Bowman, R.M. Andiman and T. Nagamoto, Exp. Neurol., 49 (1975) 115.
704. S.H. Dromgoole, D.S. Campion and J.B. Peter, Biochem. Med., 13 (1975) 307.
705. D.S. Campion, R. Olsen, S.H. Dromgoole and J.B. Peter, Exp. Neurol., 52 (1976) 216.
706. W.L. Holmes, J. Am. Oil Chem. Soc., 41 (1964) 702.
707. W.L. Holmes, N.W. DiTullio, M.B. Jones and W.H. Cevallos, Circulation, 34, Suppl. III (1966) 15.
708. N.J. Doorenbos and K.A. Kerridge, J. Heterocycl. Chem., 2 (1965) 126.
709. L.G. Donaruma and P.U. Flath, J. Med. Chem., 13 (1970) 966.
710. L.G. Donaruma and P.U. Flath, J. Med. Chem., 14 (1971) 999.
711. C.R. Craig, J. Pharm. Exp. Ther., 164 (1968) 371.
712. O. Foussard-Blanpin, Ann. Pharm. Fr., 27 (1969) 191.
713. E.C. Taylor and K. Lenard, Chem. Commun., (1967) 97.
714. K. Isomura, T. Yamazaki, M. Nagata and K. Matoba, Japan. Patent 75,05,400 (1975); Chem. Abstr., 83 (1975) 10596.
715. R.B. Conrow, E.W. Cantrall and S.Bernstein, Steroids, 9 (1967) 307.
716. K. Sasaki, U.S. Patent 3,156,710 (1964); Chem. Abstr., 62 (1965) 7837.
717. K. Sasaki, U.S. Patent 3,123,623 (1964); Chem. Abstr., 60 (1964) 12089.
718. G.H. Phillips and G. Ewan, Ger. Offen. 2,715,078 (1977); Chem. Abstr., 88 (1978) 38078.
719. G.H. Phillips, P.J. May and B.E. Ayers, Ger. Offen. 2,700,267 (1977); Chem. Abstr., 87 (1977) 184774.
720. S.K. Figdor, M.J. Kodet, B.M. Bloom, E.J. Agnello, S.Y. P'An and G.D. Lauback, J. Pharmacol. Exp. Ther., 119 (1957) 299.
721. M. Szablowska and H. Selye, Arch. Int. Pharmacodyn., 195 (1972) 351.
722. D.R. Buhler, L.C. Schroeter and W.J. Wechter, J. Med. Chem., 8 (1965) 215.
723. L. Gyermek, J. Iriarte and P. Crabbé, J. Med. Chem., 11 (1968) 117.
724. R. Mechoulam, Israel J. Chem., 6 (1968) 909.
725. O. Blanpin and A. Quevauviller, Ann. Pharm. Fr., 18 (1960) 177.
726. G.A. Overbeek and I.L. Bonta, in: Hormonal Steroids, eds. L. Martini and A. Pecile (Academic Press, New York, 1964) Vol. 1, p. 493.
727. C.L.Hewett, D.S. Savage, J.J. Lewis and M.F. Sugrue, J. Pharm. Pharmacol., 16 (1964) 765.
728. C.L. Hewett and D.S. Savage, J. Chem. Soc. (C), (1967) 582.
729. C.R. Craig, J. Pharmacol. Exp. Ther., 153 (1966) 337.
730. G. Flouret and W. Cole, J. Med. Chem., 11 (1968) 880.
731. G. Flouret, W. Cole and U. Biermacher, J. Med. Chem., 15 (1972) 1281.
732. J. Kulcsár-Gergely, Arzneim. Forsch., 26 (1976) 55.

733. M. Martin-Smith in: Drug Design, ed. E.J. Ariëns (Academic Press, New York, 1971) Vol. II, p. 453.
734. W.R. Buckett, Adv. Drug. Res. 10 (1975) 53.
735. M.M. Janot, F. Lainé and R. Goutarel, Ann. Pharm. Fr., 18 (1960) 673.
736. A. Quevauviller and F. Lainé, Ann. Pharm. Fr., 18 (1960) 678.
737. R. Goutarel, Tetrahedron, 14 (1961) 126.
738. M.M. Janot, F. Lainé, Q. Khuong-Huu and R. Goutarel, Bull. Soc. Chim. Fr. (1962) 111.
739. F. Khuong Huu-Lainé and W. Pinto-Scognamiglio, Arch. Int. Pharmacodyn. Ther., 147 (1964) 209.
740. M. Alauddin, B. Caddy, J.J. Lewis, M. Martin-Smith and M.F. Sugrue, J. Pharm. Pharmacol., 17 (1965) 55.
741. R.S. Biggs, M. Davis, R. Wien, Experientia, 20 (1964) 119.
742. D.G. Bamford, D.F. Biggs, M. Davis and E.W. Parnell, Br. J. Pharmacol. Chemother., 30 (1967) 194.
743. M. Davis, E.W. Parnell and J.R. Rosenbaum, J. Chem. Soc. (C), (1967) 1045.
744. S. Loewe and S.C. Harvey, Naunyn-Schmiedeberg's Arch. Exp. Pathol. Pharmakol., 214 (1952) 214.
745. C.J. Cavallito and A.P. Gray, Prog. Drug. Res. 2 (1960) 135.
746. P.G. Waser in: Curare and Curare-like Agents, eds. D. Bovet, F. Bovet-Nitti and G.B. Marini-Bettolo (Elsevier, Amsterdam, 1959) p. 219.
747. D.G. Bamford, D.F. Biggs, M. Davis and E.W. Parnell, J. Pharm. Pharmacol., 23 (1971) 595.
748. D. Busfield, K.J. Child, A.J. Clarke, B. Davis and M.G. Dodds, Brit. J. Pharmacol. Chemother., 32 (1968) 609.
749. W.W. Mushin and W.W. Mapleson, Br. J. Anaesth., 36 (1964) 761.
750. I.R. Verner, Proc. R. Soc. Med., 60 (1967) 1280.
751. W.L.M. Baird and A.M. Reid, Br. J. Anaesth., 39 (1967) 775.
752. T.M. Speight and G.S. Avery, Drugs, 4 (1972) 163.
753. W. Dick and R. Droh, Anaesthesist, 19 (1970) 173.
754. S.A. McDowell and R.S.J. Clarke, Anaesthesia, 24 (1969) 581.
755. W.R. Buckett, C.L. Hewett and D.S. Savage, J. Med. Chem., 16 (1973) 1116.
756. C.L. Hewett and D.S. Savage, J. Chem. Soc. (C), (1968) 1134.
757. J.J. Lewis, M. Martin-Smith, T.C. Muir and H.H. Ross, J. Pharm. Pharmacol., 19 (1967) 502.
758. D.S. Savage, A.F. Cameron, G. Ferguson, C. Hannaway and I.R. Mackay, J. Chem. Soc. (B), (1971) 410.
759. W.R. Buckett, C.E.B. Marjoribanks, F.A. Marwick and M.B. Marton, Br. J. Pharmacol. Chemother., 32 (1968) 671.
760. S.A. Feldman and M.F. Tyrrell, Anaesthesia, 25 (1970) 349.
761. J. Norman and R.L. Katz, Br. J. Anaesth., 43 (1971) 313.
762. M.F. Sugrue, N. Duff and I. Mcindewar, J. Pharm. Pharmacol., 27 (1975) 721.
763. S. Agoston, E. J. Crul, V.W. Kersten, M.C. Houwertjes and A.H.J. Scaf, Acta Anaesth. Scand., 21 (1977) 24.
764. Z. Tuba, M. Bor, S. Szeberenyi, E. Karpati, L. Szporny and G. Szepesi, Br. Patent 1,398,050 (1975); Chem. Abstr., 83 (1975) 147655.
765. J.M. Thompson, Anaesthesia, 31 (1976) 219.
766. P. Waser, Naunyn-Schmiedeberg's Arch. Exp. Pathol. Pharmakol., 279 (1973) 399.

767. H. Shindo, E. Nakajima, N. Miyakoshi and E. Shigehara, Chem. Pharm. Bull., 22 (1974) 2502.
768. S. Agoston, G.A. Vermeer, U.W. Kersten and D.K.F. Meijer, Acta Anaesth. Scand., 17 (1973) 267.
769. K. Tanaka, M. Hioki and H. Shindo, Chem. Pharm. Bull., 22 (1974) 2599.
770. W. Buzello, Anaesthesist, 24 (1975) 13.
771. A.A. Somogyi, C.A. Shanks and E.J. Triggs, Eur. J. Clin. Pharmacol., 12 (1977) 23.
772. H. Singh, D. Paul and V.V. Parashar, J. Chem. Soc. Perkin I, (1973) 1204.
773. I.G. Marshall, D. Paul and H. Singh, J. Pharm. Pharmacol., 25 (1973) 441.
774. I.G. Marshall, D. Paul and H. Singh, Eur. J. Pharmacol., 22 (1973) 129.
775. H. Singh and D. Paul, J. Chem. Soc. Perkin I, (1974) 1475.
776. A. Gandiha, I.G. Marshall, D. Paul and H. Singh, J. Pharm. Pharmacol., 26 (1974) 871.
777. A. Gandiha, I.G. Marshall, D. Paul, I.W. Rodger, W. Scott and H. Singh, Clin. Exp. Pharmacol. Physiol., 2 (1975) 159.
778. A.L. Harvey, D. Paul, I.W. Rodger and H. Singh, J. Pharm. Pharmacol., 28 (1976) 617.
779. M.A. Mazid, R.A. Palmer, H. Singh and D. Paul, Acta Crystallogr., B33 (1977) 3641.
780. H. Singh, T.R.Bhardwaj, N.K. Ahuja and D. Paul, J. Chem. Soc. Perkin I, in the press.
781. P. Teerapong, I.G. Marshall, A.L. Harvey, H. Singh, D. Paul, T.R. Bhardwaj and N.K. Ahuja, J. Pharm. Pharmacol., 29 (Suppl.) (1977) 80P.
782. I.G. Marshall and M. Martin-Smith, Euro. J. Pharmacol., 17 (1972) 39.
783. R.P. Stephenson, Br. J. Pharmacol. Chemother., 3 (1948) 237.
784. J.L.P. Mainil, U.S. Patent 3,098,859 (1963); Chem. Abstr., 60 (1964) 642.
785. W.R. Buckett, C.L. Hewett, F.A. Marwick and D.S. Savage, Chim. Ther., 2 (1967) 164.
786. O. Jeger and H.U. Wehrli, Swiss Patent 538,511 (1973); Chem. Abstr., 79 (1973) 137385.
787. O. Jeger and H.U. Wehrli, Swiss Patent 537,948 (1973); Chem. Abstr., 79 (1973) 137387.
788. G. Bengtsson, S. Ullberg, N. Wiquist and E. Diczfaluzy, Acta Endocrinol., 46 (1964) 544.
789. K.R. Laumas and A. Farooq, Int. Congr. on Hormonal Steroids, 2nd, Miland, 1966, Abstr. Excerpta Medica Foundation, New York, 1966, p. 274.
790. R.E. Counsell, B.H. Hong, R.E. Willette and V.V. Ranade, Steroids, 11 (1968) 544.
791. W.J. Lossow, G. Shymala, S. Shah and I.L. Chaikoff, Proc. Soc. Exp. Biol. Med., 119 (1965) 126.
792. J.H. Gans, Lipids, 3 (1968) 324.
793. L.E. Appelgren, Acta Physiol. Scand., 71 Suppl. 301 (1967) 1.
794. T. Nagai, B.A. Solis and C.S. Koh, J. Nucl. Med., 9 (1968) 576.
795. R.E. Counsell, V.V. Ranade, R.J. Blair, W.H. Beierwaltes and P.A. Weinhold, Steroids, 16 (1970) 317.
796. L.M. Lieberman, W.H. Beierwaltes, J.W. Conn, A.N. Ansari and H. Nishiyama, New Engl. J. Med., 285 (1971) 1387.
797. W.H. Beierwaltes, L.M. Lieberman, A.N. Ansari and H. Nishiyama, J. Am. Med. Assoc., 216 (1971) 275.
798. R.J. Blair, W.H. Beierwaltes, L.M. Lieberman, C.M. Boyd, R.E. Counsell, P.A. Weinhold and V.M. Varma, J. Nucl. Med., 12 (1971) 176.
799. J.W. Conn, R. Morita, E.L. Cohen, W.H. Beierwaltes, W.J. McDonald and K. Herwig, Arch. Int. Med., 129 (1972) 417.
800. D.E. Schteingart, J.W. Conn, L.M. Lieberman and W.H. Beierwaltes, Arch. Int. Med., 130 (1972) 384.
801. R. Morita, L.M. Lieberman, W.H. Beierwaltes, J.W. Conn, A.N. Ansari and H. Nishiyama, J. Clin. Endocrinol. Metab., 34 (1972) 36.

802. D.C. Moses, D.E. Schteingart, M.F. Sturman, W.H. Beierwaltes and R.D. Ice, Surg. Gynecol. Obstet., 139 (1974) 201.
803. W.H. Beierwaltes, M.F. Sturman, U. Ryo and R.D. Ice, J. Nucl. Med., 15 (1974) 246.
804. M.F. Sturman, D.C. Moses, W.H. Beierwaltes, T.S. Harrison, R.D. Ice and R.P. Dorr, Surg. Gynecol. Obstet., 138 (1974) 177.
805. W.H. Beierwaltes, M.F. Sturman, U. Ryo and R.D. Ice, J. Nucl. Med., 15 (1974) 246.
806. J.E. Seabold, E.L. Cohen, W.H. Beierwaltes, D.L. Hinerman, R.H. Nishiyama, J.J. Bookstein, R.D. Ice and S. Balachandran, J. Clin. Endocrinol. Metab., 42 (1976) 41.
807. M.J. Hogan, J.McRae, M. Schambelan and E.G. Biglieri, New Engl. J. Med., 294 (1976) 410.
808. H. Kehlet, M. Blichert-Toft, S. Hancke, J.F. Pedersen, J.K. Kristensen, F. Efsen, H. Dige-Petersen, J. Fogh, K. Lockwood and E. Hasner, Br. Med. J., 2 (1976) 665.
809. M. Kojima, M. Maeda, H. Ogawa, K. Nitta and T. Ito, J. Chem. Soc. Chem. Commun., (1975) 47.
810. M. Maeda, M. Kojima, H. Ogawa, K. Nitta and T. Ito, Steroids, 26 (1975) 241.
811. G.P. Basmadjian, K.R. Hetzel, R.D. Ice and W.H. Beierwaltes, J. Labelled Compounds, 11 (1975) 427.
812. M.W. Couch, K.N. Scott and C.M. Williams, Steroids, 27 (1976) 451.
813. K.N. Scott, M.W. Couch, T.H. Mareci and C.M. Williams, Steroids, 28 (1976) 295.
814. M. Kojima, M. Maeda, H. Ogawa, K. Nitta and T. Ito, J. Nucl. Med., 16 (1975) 666.
815. K. Nitta, H. Ogawa, T. Ito, M. Kojima and M. Maeda, Chem. Pharm. Bull., 24 (1976) 2322.
816. S.D. Sarkar, W.H. Beierwaltes, R.D. Ice, G.P. Basmadjian, K.R. Hetzel, W.P. Kennedy and M.M. Mason, J. Nucl. Med., 16 (1975) 1037.
817. S. Fukuchi, K. Nakajima, T. Miura, K. Haruyama, M. Nakamura, H. Ogawa and K. Nitta, Clin. End. (Japan), 23 (1975) 1051.
818. K. Matsuura, I. Kamoi, K. Watanabe, C. Nakayama and I. Koga, Radioisotopes, 24 (1975) 780.
819. S.D. Sarkar, E.L. Cohen, W.H. Beierwaltes, R.D. Ice, S.P. Gill and E.N. Gold, J. Nucl. Med., 17 (1976) 563.
820. M. Maeda, H. Komatsu, M. Kojima and H. Ogawa, Chem. Pharm. Bull., 24 (1976) 1398.
821. M. Kojima, M. Maeda, H. Komatsu, H. Shimoirisa, H. Ogawa, K. Nitta and T. Ito, Steroids, 29 (1977) 443.
822. S.D. Sarkar, R.D. Ice, W.H. Beierwaltes, S.P. Gill, S. Balachandran and G.P. Basmadjian, J. Nucl. Med., 17 (1976) 212.
823. R.W. Scot Skinner, R.V. Pozderac, R.E. Counsell, C.-F. Hsu and P.A. Weinhold, Steroids, 30 (1977) 15.
824. G.A.D. Haslewood, Biochem, J., 35 (1941) 1307.
825. P. Mantegazza and R. Tommasini, Atti Soc. Lombarda Sci. Med. Biol., 7 (1952) 496; Chem. Abstr., 47 (1953) 5557.
826. P. Mantegazza and R. Tommasini, Boll. Soc. Ital. Biol. Sper., 27 (1951) 631; Chem. Abstr., 48 (1954) 866.
827. G. Cavallini, E. Massarani and D. Nardi, J. Med. Chem., 7 (1964) 673.
828. J. Barnett, B.E. Ryman and F. Smith, J. Chem. Soc., (1946) 524.
829. S.P. James, F. Smith, M. Stacey and M. Webb, J. Chem. Soc., (1946) 665.
830. M.L. Hilton and M. Webb, J. Chem. Soc., (1951) 2767.
831. M.L. Hilton, A.S. Jones and J.R.B. Westwood, J. Chem. Soc., (1955) 3449.

832. J. Barnett, B.E. Ryman and F. Smith, J. Chem. Soc., (1946) 528.
833. H.L. Herzog, C.C. Payne and E.B. Hershberg, J. Am. Chem. Soc., 77 (1955) 5324.
834. D.P. Dodgson and R.D. Haworth, J. Chem. Soc., (1952) 67.
835. R.A. Micheli and C.K. Bradsher, J. Am. Chem. Soc., 77 (1955) 4788.
836. R. Varela and F.A. Kincl, Ciencia, 21 (1962) 213.
837. Teikoku Hormone Mfg. Co., Ltd., Japan, Patent 12,231 (1960); Chem. Abstr., 55 (1961) 11470.
838. Y. Hirami, R. Ohuchi, Y. Kurosawa and H. Mori, Agr. Biol. Chem., 39 (1975) 843; Chem. Abstr., 83 (1975) 59140.
839. C. Rufer, Justus Liebigs Ann. Chem., 726 (1969) 145.
840. L.D. Boeck, M.M. Hoehn, J.E. Westhead, R.K. Wolter and D.N. Thomas, J. Antibiot., 28 (1975) 95.
841. R.S. Gordee and T.F. Butler, J. Antibiot., 28 (1975) 112.
842. J.W. Chamberlin, M.O. Chaney, S. Chen, P.V. Demaco, N.D. Jones and J.L. Occolowitz, J. Antibiot., 27 (1974) 992.
843. R.B. Bailey, P.R. Hays and L.W. Parks, J. Bacteriol., 128 (1976) 730.
844. C.D. Jones, U.S. Patent 4,001,246 (1977); Chem. Abstr., 87 (1977) 53493.
845. C.D. Jones, U.S. Patent 4,008,238 (1977); Chem. Abstr., 86 (1977) 171737.
846. J.W. Chamberlin, U.S. Patent 4,039,547 (1977); Chem. Abstr., 88 (1978) 38080.
847. J.G. Morgan, K.D. Berlin, N.N. Durham and R.W. Chesnut, J. Org. Chem., 36 (1971) 1599.
848. R.W. Chesnut, N.N. Durham, R.A. Brown, E.A. Mawdsley and K.D. Berlin, Steroids, 27 (1976) 525.
849. R.W. Chesnut, D.F. Haslam, K.D. Berlin, J.G. Morgan and N.N. Durham, Bacteriol. Proc., (1971) 7.
850. M.L. Higgins, R.W. Chesnut, F.R. Leach, J.G. Morgan, K.D. Berlin and N.N. Durham, Steroids, 19 (1972) 301.
851. R.W. Chesnut, M.L. Higgins, F.R. Leach, J. Robinson, K.D. Berlin and N.N. Durham, Steroids, 28 (1976) 535.
852. J.G. Morgan, K.D. Berlin, N.N. Durham and R.W. Chesnut, J. Heterocycl. Chem., 8 (1971) 61.
853. N.N. Durham, R.W. Chesnut, D.F. Haslam and K.D. Berlin, Ann. Okla. Acad. Sci., (1974) 77.
854. R.W. Ghesnut, N.N. Durham, E.A. Mawdsley, G.X. Thyvelikakath, K.D. Berlin and B.M. Fung, Biochem. J., 143 (1974) 789.
855. R.W. Chesnut, D.F. Haslam, N.N. Durham and K.D. Berlin, Can. J. Biochem., 50 (1972) 516.
856. M.M. Hashem, K.D. Berlin, R.W. Chesnut and N.N. Durham, J. Carbohydr. Nucleos. Nucleot., 2 (1975) 357.
857. M.M. Hashem, K.D. Berlin, R.W. Chesnut and N.N. Durham, J. Med. Chem., 19 (1976) 229.
858. E.A. Mawdsley, K.D. Berlin, R.W. Chesnut and N.N. Durham, J. Med. Chem., 19 (1976) 239.
859. K. Ramalingam, L.F. Wong, K.D. Berlin, R.A. Brown, R. Fischer, J. Blunk and N.N Durham, J. Med. Chem., 20 (1977) 664.
860. R.F. Smith, D.E. Shay and N.J. Doorenbos, J. Bacteriol., 85 (1963) 1295.
861. R.F. Smith, D.E. Shay and N.J. Doorenbos. Appl. Microbiol., 11 (1963) 542.
862. F. Varricchio, N.J. Doorenbos and A. Stevens, J. Bacteriol., 93 (1967) 627.

863. N.J. Doorenbos and P.C. Bossle, J. Pharm. Sci., 54 (1965) 1691.
864. R.F. Smith, D.E. Shay and N.J. Doorenbos, J. Pharm. Sci., 53 (1964) 1214.
865. W.E. Solomons and N.J. Doorenbos, J. Pharm. Sci., 63 (1974) 19.
866. N.J. Doorenbos and J.C. Kim, J. Pharm. Sci., 63 (1974) 620.
867. N.J. Doorenbos and J.M. Brown, J. Pharm. Sci., 60 (1971) 1234.
868. N.J. Doorenbos and W.E. Solomons, J. Pharm. Sci., 62 (1973) 638.
869. N.J. Doorenbos, S.S. Vaidya and R.C. Havranek, Chem. Ind., (1967) 704.
870. N.J. Doorenbos, J. Scott and S.S. Vaidya, J. Pharm. Sci., 60 (1971) 1236.
871. R.M. Scribner, U.S. Patent 3,337,535 (1967); Chem. Abstr., 67 (1967) 117115.
872. R.M. Scribner, U.S. Patent 3,342,808 (1967); Chem. Abstr., 67 (1967) 117116.
873. W. Nagata and S. Hirai, Chem. Pharm. Bull., 16 (1968) 1550.
874. E.F. Elslager in: Medicinal Chemistry Part I, ed. A. Burger (Wiley-Interscience, New York, 1970) p. 522.
875. G.W. Irving, Jr., T.D. Fontaine and S.P. Doolittle, J. Bacteriol., 52 (1945) 601.
876. G. Schuster and A. Tarrade, Fed. Proc., 28 (1969) 264.
877. L.A. Mitscher, J.V. Juvarkar and J.L. Beal, Experientia, 32 (1976) 415.
878. G.R. Vavasour, H.I. Bolker and A.F. McKay, Can. J. Chem., 30 (1952) 933.
879. G.G. Hazen, Dissertation Abstr., 12 (1952) 449.
880. W.J. Gensler and G.M. Sherman, J. Org. Chem., 23 (1958) 1227.
881. R.E. Havranek and N.J. Doorenbos, J. Am. Pharm. Assoc. Sci. Ed., 49 (1960) 328.
882. S.H. Burstein and H.J. Ringold, J. Org. Chem., 26 (1961) 3084.
883. G.V. Rao and C.C. Price, J. Org. Chem., 27 (1962) 205.
884. C.R. Walk, T.C. Chou and H.H. Lin, J. Med. Chem., 10 (1967) 255.
885. J.B. Jones, D.J. Adam and J.D. Leman, J. Med. Chem., 14 (1971) 827.
886. M.H. Benn, A.M. Creighton, L.N. Owen and G.R. White, J. Chem. Soc., (1961) 2365.
887. T. Nogrady, K.M. Vagi and V.W. Adamkiewics, Can. J. Chem., 40 (1962) 2126.
888. I. Niculescu-Duuáz, A. Cambanis and E. Târnâuceanu, J. Med. Chem., 10 (1967) 172.
889. E. Cioranescu and D. Raileanu, Acad. Repub. Pop. Rom. Stud. Cercet. Chim., 10 (1962) 295.
890. D. Raileanu and E. Cioranescu, Acad. Repub. Prop. Rom. Stud. Cercet. Chim., 11 (1963) 433.
891. H. Zimmel and A. Macrineanu, Oncol. Radiol., 5 (1964) 425.
892. A.H. El Masry, V.C. Braun, C.J. Nielsen and W.B. Pratt, J. Med. Chem., 20 (1977) 1134.
893. J. Müntzing, S.K. Shukla, T.M. Chu, A. Mittelman and G.P. Murphy, Invest. Urol., 12 (1974) 65.
894. A. Mittelman, S.K. Shukla, K. Welvaart and G.P. Murphy, Cancer Chemother. Rep., 59 (1975) 219.
895. T. Nilsson and G. Jonsson, Cancer Chemother. Rep., 59 (1975) 229.
896. D.D. von Hoff, M. Rozencweig, M. Slavik and F.M. Muggia, J. Urol., 117 (1977) 464.
897. R.Y. Kirdani, J. Müntzing, M.J. Varkarakis, G.P. Murphy and A.A. Sandberg, Cancer Res., 34 (1974) 1031.
898. P.A. Hoisaeter, Acta Endocrinol., 80 (1975) 188.
899. R.Y. Kirdani, A. Mittelman, G.P. Murphy and A.A. Sandberg, J. Clin. Endocrinol. Metab., 41 (1975) 305.
900. J.M. McMillin, U.S. Seal and R.P. Doe, Invest. Urol., 15 (1977) 151.
901. M.J. Varkarakis, R.Y. Kirdani, G.P. Murphy and A.A. Sandberg, Proc. Soc. Exp. Biol. Med., 148 (1975) 904.

902. L.F. Larionov, S.A. Degeteva and N.A. Lesnaya, Vopr. Onkl., 8 (1962) 12.
903. S.A. Degeteva, Vopr. Onkl., 10 (1964) 52.
904. S.A. Degeteva and L.F. Larionov, Vopr. Onkl., 12 (1966) 51.
905. E.N. Shkodinskaya, E.M. Kurdyukova, O.S. Vasina and A. Ya Berlin, J. Gen. Chem. USSR, 32 (1962) 945.
906. F.J. Ansfield, A.C. Carter, I.S. Goldenberg and A. Segaloff, Cancer Chemother. Rep., 55 (1971) 259.
907. E.P. Vollmer, D.J. Taylor, I.J. Masnyk, D. Gooney, B. Levine, C. Piczak and L. Trench, Cancer Chemother. Rep., Part 3, 4 (1973) 103.
908. M.E. Wall, G.S. Abernethy Jr., F.I. Carroll and D.J. Taylor, J. Med. Chem., 12 (1969) 810.
909. F.I. Carroll, A. Philip, J.T. Blackwell, D.J. Taylor and M.E. Wall, J. Med. Chem., 15 (1972) 1158.
910. E.P. Vollmer, D.J. Taylor, I.J. Masnyk, D. Gooney, B. Levine and C. Piczak, Cancer Chemother. Rep., Part 3, 4 (1973) 121.
911. P. Catsoulacos and L. Boutis, Eur. J. Med. Chem. 9 (1974) 211.
912. G.L. Wampler and P. Catsoulacos, Cancer Treat. Rep., 61 (1977) 37.
913. P. Catsoulacos, L. Boutis and K. Dimitropoulos, Eur. J. Med. Chem., 11 (1976) 189.
914. P. Catsoulacos and L. Boutis, Cancer Chemother. Rep., 57 (1973) 365.
915. E.L. Foster and R.T. Blickenstaff, J. Med. Chem., 11 (1968) 1106.
916. A.H. Evenaar, E.H.R. Wins and L.M. van Putten, Eur. J. Cancer, 9 (1973) 773.
917. L. Brandt, I. Könyves and T.R. Möller, Acta Med. Scand., 197 (1975) 317.
918. T.R. Möller, L. Brandt, I. Könyves and L.G. Lindberg, Acta Med. Scand., 197 (1975) 323.
919. L. Brandt and I. Könyves, Eur. J. Cancer, 13 (1977) 393.
920. K.R. Harrap, P.G. Riches, E.D. Gilby, S.M. Sellwood, R. Wilkinson and I. Könyves, Eur. J. Cancer, 13 (1977) 873.
921. E.L. Foster and R.T. Blickenstaff, Steroids, 24 (1974) 737.
922. E.L. Foster and R.T. Blickenstaff, Steroids, 27 (1976) 353.
923. F. Schenider, J. Hamsher and R.E. Beyler, Steroids, 8 (1966) 553.
924. A. Segaloff, J.B. Weeth, E.L. Rongone, P.G. Murison and C.Y. Bowers, Cancer, 13 (1960) 1017.
925. A. Segaloff, J.B. Weeth, K.K. Meyer, E.L. Rongone and M.E.G. Cuningham, Cancer, 15 (1962) 633.
926. M. Van Rymenant, A. Coune, H.J. Tagnon and S. Simon, Acta Clin. Belg., 18 (1963) 469.
927. T.J. Cantino and G.S. Gordan, Cancer, 20 (1967) 458.
928. H. Volk, R.H. Deupree, I.S. Goldenberg, R.C. Wilde, R.A. Carabasi and G.C. Escher, Cancer, 33 (1974) 9.
929. A.N. Papaioannou and H. Volk, Cancer Chemother. Rep., 50 (1966) 323.
930. E.L. Rongone, A. Segaloff, J. Fried and E.F. Sabo, J. Biol. Chem., 236 (1961) 2624.
931. A. Segaloff, R.B. Gabbard and B.T. Carriere, Steroids, 7 (1966) 321.
932. A. Segaloff, K.K. Meyer, E.L. Rongone, J.B. Weeth and M. Cuningham, Cancer, 15 (1962) 636.
933. I. Barta, K. Albecht, G. Ambrus, E. Csanyi, J. Gyimesi and E. Kiraly, Hung. Teljes, 10,698 (1975); Chem. Abstr., 84 (1976) 105907.
934. W.A. Fish, W.M. Stohs and O.P. Hichey, Cancer Res., 14 (1954) 433.
935. E. Schwenk and D.F. Stevens, Cancer Res., 18 (1958) 193.

936. H. Lettré in: Experimental Tumor Research, ed. F. Hamburger (Karger, Basel, 1960) Vol. 1, p. 329.
937. H. Lettré and L. Knof, Ger. Patent 1,118,197 (1959); Chem. Abstr., 56 (1962) 10252.
938. H. Mori, V.S. Gandhi and E. Schwenk, Chem. Pharm. Bull., 10 (1962) 842.
939. D.F. Stevens and E. Schwenk, Naturwissenschaften, 45 (1958) 218.
940. R. Fumagalli, E. Grossi-Paoletti, P. Paoletti and R. Paoletti, J. Neurochem., 13 (1966) 1005.
941. J. Buckingham, G.J.F. Chittenden and R.D. Guthrie, J. Chem. Soc. (C) (1967) 1703.
942. H. Lettré, A. Jahn and R. Pfirrmann, Justus Liebig's Ann. Chem., 615 (1958) 222.
943. H. Lettré, Prog. Exp. Tumor Res., 1 (1960) 329.
944. S.S. Legha and S.K. Carter, Cancer Treat. Rev., 3 (1976) 205.
945. K. Yamaguchi, N. Uchida and H. Kasai, Ann. Rep. Shionogi Res. Lab., 20 (1970) 96.
946. A. Matsuzawa and T. Yamamoto, Cancer Res., 36 (1976) 1598.
947. Japanese Cooperative Group of Hormonal Treatment for Breast Cancer (Chairman: M. Fujimori), Cancer, 31 (1973) 789.
948. S. Kumaoka, O. Takatani, M. Yoshida, S. Miura, T. Takao, Y. Hamanaka, M. Izuo and T. Okada, Japan. J. Clin. Oncol., 6 (1974) 65.
949. A. Matsuzawa and T. Yamamoto, Cancer Res., 37 (1977) 4408.
950. K. Shida, J. Shimazaki, E. Urano, H. Kurihard, H. Takahoshi, N. Furuya and M. Taya, Japan. J. Urol., 63 (1972) 109.
951. M.L. Tainter, A. Arnold, A.L. Beyler, H.P. Drobeck, G.O. Potts and C.H. Roth, J. Am. Ger. Soc., 11 (1963) 1140.
952. E. Bonmossar, F. Melan and A. Montagnani-Marelli, Arch. Ital. Pat. Clin. Tumori, 10 (1967) 37; Cancer Chemother. Abstr., 8 (1967) 2069.
952a. S. Muntoni, Nature (London), 215 (1967) 969.
953. S. Muntoni and B. Loddo, Boll. Soc. Ital. Biol. Sper., 43 (1967) 1257; Cancer Chemother. Abstr., 8 (1967) 1185.
954. S. Muntoni and B. Loddo, Boll. Soc. Ital. Biol. Sper., 43 (1967) 1260; Cancer Chemother. Abstr., 8 (1967) 1186.
955. J.E. Pike, J.E. Grady, J.S. Evans and C.G. Smith, J. Med. Chem., 7 (1964) 348.
956. K.-H. Lee, T. Ibuka, S.-H. Kim, B.R. Vestal, I.H. Hall and E.-S. Huang, J. Med. Chem., 18 (1975) 812.
957. M.E. Kuehne, E.A. Konopka and B.F. Lambert, J. Med. Pharm. Chem., 5 (1962) 281.
958. D.S. Savage, A. Emminger and U. Mohr, Cancer Lett., 2 (1977) 267.
959. A. Emminger, E. Kahmann and D.S. Savage, Cancer Lett., 2 (1977) 273.
960. A. Emminger and D.S. Savage, Cancer Lett., 3 (1977) 157.
961. R.T. Blickenstaff and E.L. Foster, J. Org. Chem., 26 (1961) 5029.
962. K.H. Kwan, W.I. Higuchi, A.M. Molokhia and A.F. Hofmann, J. Pharm. Sci., 66 (1977) 1105.
963. D. Mufson, K. Triyanond and L.J. Ravin, J. Pharm. Sci., 64 (1975) 362.
964. M. Morisaki, H. Ohotaka, M. Okubayashi, N. Ikekawa, Y. Horie and S. Nakasone, J. Chem. Soc. Chem. Commun., (1972) 1275.
965. M. Morisaki, H. Ohotaka, N. Awata, N. Ikekawa, Y. Horie and S. Nakasone, Steroids, 24 (1974) 165.
966. J.P. Allais, A. Alcaide and M. Barbier, Experientia, 29 (1973) 944.
967. P.J. Randall, J.G.L. Jones, I.F. Cook, H.H. Rees and T.W. Goodwin, J. Chem. Soc. Chem. Commun., (1972) 1296.

968. Y. Fujimoto, M. Morisaki, N. Ikekawa, Y. Horie and S. Nakasone, Steroids, 24 (1974) 367.
969. C.-C. Chin and J.C. Warren, J. Biol. Chem., 243 (1968) 5056.
970. T.G. Muldoon and J.C. Warren, J. Biol. Chem., 244 (1969) 5430.
971. C.-C. Chin and J.C. Warren, Biochemistry, 9 (1970) 1917.
972. P.J. Guthrie, Can. J. Chem., 50 (1972) 3993.
973. P.J. Guthrie and Y. Ueda, J. Chem. Soc. Chem. Commun., (1973) 898.
974. J. McKenna, J.M. McKenna and D.W. Thornthwaite, J. Chem. Soc. Chem. Commun., (1977) 809.
975. H.R. Mahler and G. Dutton, J. Mol. Biol., 10 (1964) 157.
976. H.R. Mahler, R. Goutarel, Q. Khuong-Huu and M.T. Ho, Biochemistry, 5 (1966) 2177.
977. H.R. Mahler, G. Green, R. Goutarel and Q. Khuong-Huu, Biochemistry, 7 (1968) 1568.
978. R. Glaser and E.J. Gabbay, J. Org. Chem., 35 (1970) 2907.
979. E.J. Gabbay and R. Glaser, Biochemistry, 10 (1971) 1665.

This Page Intentionally Left Blank

Progress in Medicinal Chemistry — Vol. 16, edited by G.P. Ellis and G.B. West

3 The Molecular Basis for the Action of Some DNA-Binding Drugs

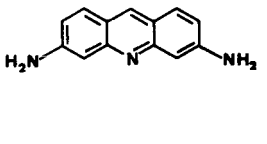
STEPHEN NEIDLE, B.Sc., Ph.D.

Department of Biophysics, University of London King's College, 26-29 Drury Lane, London WC2B 5RL, England

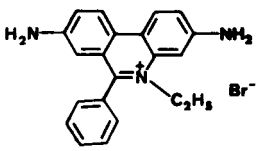
INTRODUCTION

It is now well established that a large number of drugs, carcinogens and mutagens exert their effect primarily by interacting directly with the genetic material of cells, *i.e.*, with nuclear DNA [1—4]. This is considered to be the most sensitive target in the cell for such attack [5]. In so doing, these compounds impair the ability of DNA to act as a template for the processes of nucleic acid expression and synthesis — hence a diminuition (or even complete cessation) of cell growth is produced, with even cell death as an eventual outcome in some instances. Many of these compounds are believed to bind covalently to nucleic acids (*cf.* the benzpyrene carcinogens, the platinum anti-tumour agents), whilst others interact *via* various types of non-bonded forces. This review is exclusively concerned with drugs from this latter class. Many of these have been the subject of intensive and increasing study over the past two decades for two principal reasons; firstly, they have proved to be powerful probes of nucleic acid structure and function, and secondly, a number of them have clinically useful chemotherapeutic properties, of which anti-cancer activity is most prominent. The social and economic impetus for developing effective agents with this property in particular has without doubt been a dominant factor in both funding research in this area and influencing its course of direction.

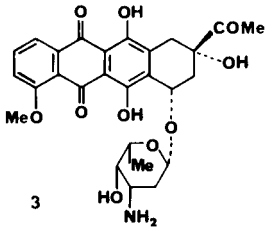
The drugs to be considered in this review exhibit a wide spectrum of biological activity, ranging from the bacteriostatic properties of the acridines such as proflavine (1) and the trypanocidal properties of ethidium bromide (homidium bromide, 2) to the anti-leukemic action of daunomycin (3). At the



1



2



3

present time, it is not clear why a particular cell type or organism is especially susceptible to any one of these drugs rather than to another; they have been found to be relatively non-specific in their ability to bind differing sequences of nucleotides in a genome. Explanations for such behaviour are most likely to be sought by considerations of pharmacokinetic and related effects. As yet, relatively little is known about molecular aspects of membrane structure and function in relation to these problems.

Considerations of nucleic acids especially DNA, as the major receptor for these drugs, have been frequently highly dependent upon the fact that there exists a large body of detailed structural and physico-chemical information on these macromolecules, a virtually unique situation for drug receptors. This in turn has enabled the interactions to be defined to a level unsurpassed by almost all other classes of drugs and antibiotics (although this level is by no means as profound as was once thought). At this point, it is relevant to summarise aspects of nucleic acid structure for those readers unfamiliar with this topic.

THE STRUCTURES OF DNA AND RNA

A combination of X-ray analysis of oriented fibres and molecular model-building established the structure of DNA to consist of two intertwined polynucleotide chains forming a double helix, which is largely held together by hydrogen-bonding (*Figure 3.1*) between specific bases — the Watson-Crick

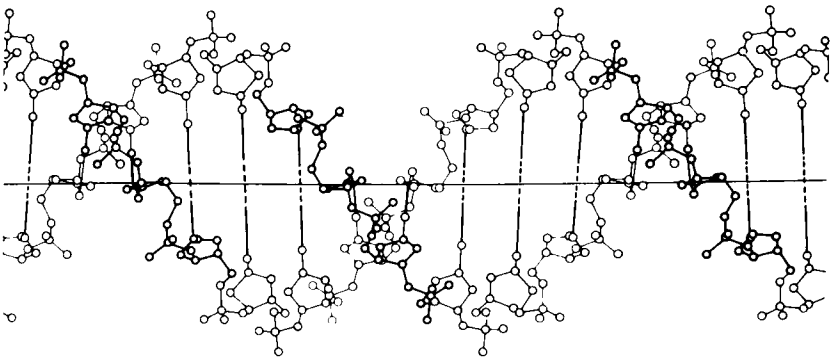
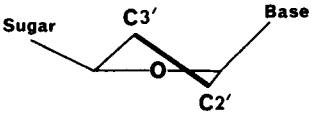


Figure 3.1. The molecular structure of DNA, viewed at right angles to the helix axis (Courtesy of M.H.F. Wilkins).



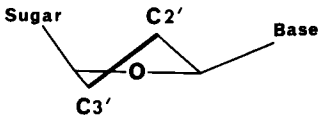


*Figure 3.2. The two principal sugar conformations of nucleic acids. (a) C2′-*endo*, (b) C3′-*endo*.*

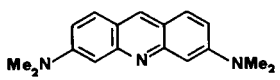
base pairs [6,7]. Detailed analysis has shown that a number of structural polymorphs exist, and these fall into two distinct conformational classes termed A and B [8]. A-DNA has C3′-*endo* puckering of the deoxyribose ring, whereas the B-form has C2′-*endo* (or C3′-*exo*) puckering (*Figure 3.2*). The former has eleven residues per complete helical turn compared with ten for the B form, which also has the bases only slightly tilted from orthogonality with the helix axis. The A→B structural transition is dependent upon the ionic strength of the medium, with the B form being considered the most likely

conformation under physiological conditions. Detailed parameters for both forms have been published [9,10]. Those fibre diffraction analyses result in the representation of an averaged polynucleotide chain, and subtle differences are not observed. Recent analyses of fibres of synthetic DNAs with repeated di- and trinucleotide sequences have shown that nucleotide sequence differences may well promote peculiarities in structural behaviour. Thus, poly(dA—dT)·poly(dA—dT) [a double-stranded copolymer of deoxyadenosine (dA) and deoxythymidine (dT) with each strand having alternating sequence] forms an 8-fold double helix [11], which nonetheless is considered to be a B-class type structure. It may well be the case that the more complex DNA-binding drugs are able to recognise differing tracts of sequence by virtue of their secondary structural differences. This notion is discussed further in a later section. It has been suggested that nucleotide sequence is the important factor in determining structural behaviour, rather than composition or repetitiveness [8]. Thus, oligo d(G)·oligo d(C) (a double stranded oligomer length with one strand of deoxyguanosines and the other of deoxycytidines) tracts tend to have rather more A than B-DNA character, with the reverse being true for oligo d(A)·oligo d(T) ones.

Double-helical RNA, in contrast, only appears to exist in one structural type, which is a variant on A-DNA [12]. This inability of RNA to undergo a transition to a B-type structure is most likely a consequence of the presence of the 2′-hydroxyl group on the sugar compared with the deoxy polymer. Thus, results obtained for drug binding to RNAs either natural or synthetic, must be interpreted with considerable caution when extrapolated to the DNA situation.

THE INTERCALATION HYPOTHESIS

The ability of acridines (particularly acridine orange (4)) to stain nucleic acids in cells has been known and exploited by cell biologists for many years [13,14]. The advent of the double-helical hypothesis for DNA structure provided the basis for a structural interpretation of these histological phenomena, as well as of many other biological properties of acridines. The classic investigations of Lerman [15—17] established that interaction of proflavine and acridine



4

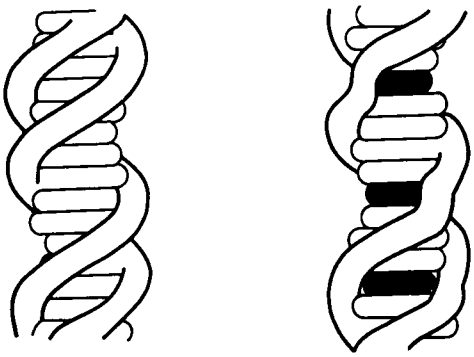


Figure 3.3. The intercalation model of Lerman [20]. The left-hand side represents native DNA, with the base pairs represented as end-on-viewed discs. The right-hand side represents drug (shaded-in discs) intercalated in-between base pairs.

orange with DNA *in vitro* produced a number of physical changes in the nucleic acid. Thus, a marked enhancement of viscosity was observed, together with a decrease in sedimentation coefficient. This suggested that the mass per unit length of the DNA molecule was diminished, and that its rod-like character was increased on drug binding. X-ray scattering data confirmed this and also showed that the 3.4 Å spacing along the helix axis was retained. The amino group reactivity of, say, proflavine, was substantially diminished on binding [18].

Lerman suggested that the common structural characteristic of all these compounds provided the basis for their mode of binding—they all possess a system of planar fused (aromatic) rings similar in dimensions to a base pair. In accordance with the observation [17] that the drugs are bound with their molecular axes perpendicular to the helix axis, Lerman propounded the hypothesis that the planar drug molecules become sandwiched (intercalated) in-between adjacent base pair (*Figure 3.3*). Model-building studies showed [15,19] that this can be accomplished by a stereochemically-feasible local unwinding of the polynucleotide backbone at the intercalation site, so that the base pairs at this site became 6.8 Å instead of 3.4 Å apart. The intercalation theory has subsequently contributed significantly to an understanding of both the detailed physico-chemical binding behaviour of many nucleic acid binding drugs and of their biological properties [20]. However, only recently has attention been focussed upon the precise details of the molecular structures of the complexes involved.

PROFLAVINE AND OTHER ACRIDINES

BINDING TO DNA

The interaction of proflavine with DNA was first studied thoroughly by Peacocke and Skerrett [21], using both a spectrophotometric method and equilibrium dialysis. The former technique in particular has been utilised in many studies of drug-DNA binding; it relies on the systematic spectral changes undergone by the drug chromophore on binding. Typically, both a bathochromic shift and a hypochromic effect are observed. Both these methods, as well as others employed in subsequent studies [22], produce closely similar binding curves which are invariably non-linear, indicating a heterogeneity of binding sites — this is confirmed by the non-linearity of the corresponding Scatchard plots. (These essentially apply the law of mass action to determine binding constants and the number of binding sites per nucleotide). These results have been interpreted in terms of two binding processes: (1) a strong one at low drug levels, up to a ratio of about 0.2 molecule of proflavine per nucleotide, followed by (2) a weaker interaction, up to the electroneutrality limit of one drug molecule per nucleotide. The binding constants do vary with ionic strength, with extent of binding in general being diminished by increasing ionic strength — process (2) being affected the most [23], indicating that it has a greater electrostatic component. The strong binding has an association constant of about 3×10^6 mol^{-1}, whereas that for process (2) is about an order of magnitude less. These observations have been interpreted as arising from an initial intercalative process, which would be expected to be stabilised by stacking forces, followed by binding of proflavine cations on the exterior of the double helix. This latter step is largely electrostatic in nature, involving interactions with negatively-charged phosphate oxygen and would be expected to be weak. It has been suggested that acridines not only aggregate but self-aggregate extensively [24] around nucleic acids; indeed, many acridines (notably proflavine itself) dimerise both in solution [25] and in the solid state [26], *via* chromophore stacking. A study [27] of the optical properties of the proflavine-DNA complex has investigated the induced optical activity of acridines on binding and has shown that T2 bacteriophage DNA has much weaker external binding. This is presumed to be due to T2 DNA being glucosylated — thus stacking of proflavine occurs normally in the large groove of the DNA double helix (which is sterically blocked in T2 DNA). Further evidence for intercalation corresponding to strong binding comes, for example, from a calorimetric and equilibrium dialysis study [28], as well as use of a brominated polynucleotide in a spin-orbital probe analysis [29].

Direct evidence for intercalation has come from the observation of lengthening of the T2 phage DNA molecule in the presence of proflavine, using the technique of autoradiography [30]. The length of the DNA is increased from about 50μm to up to 72μm. This maximum corresponds to 44% of the potential spaces between base pairs being occupied, which is the expected random maximum when adjacent sites are unoccupied *i.e.* when there is neighbour exclusion. This principle has been considered by many investigators to be of fundamental importance in considerations of drug binding [31]; however in view of recent data from diacridine-DNA binding (see later section), it is not clear whether neighbour exclusion is universal or indeed whether it is true at all. Information about the preferential binding of proflavine to particular sequences of bases may well be relevant; as yet, sufficient data are not yet available for firm conclusions to be made.

Hydrodynamic studies on sonicated DNA-proflavine complexes [32] have confirmed the earlier investigations of Lerman [15], and have shown that the length of the macromolecule increases with an increasing ratio of proflavine to nucleotide. This lengthening, rather than a stiffening of the double helix, is the cause of the increased viscosity produced by drug binding.

BINDING TO SUPERHELICAL DNA

Changes in sedimentation coefficient have also been used to monitor the binding of intercalative drugs to closed circular DNA [33]. The DNA of a number of viruses and bacteriophages, as well as that of animal mitochondria, exists not in a linear form, but linked together at each end. The double helical nature of the two strands forces the structure to be twisted into a number of constrained supercoils. Since intercalation necessarily increases DNA pitch, it would also alter the number of supercoils. *Figure 3.4* shows a sedimentation profile as increasing quantities of proflavine become bound — initially the number of right-handed supercoils decreases, with a fall in sedimentation coefficient. After a minimum point is reached, this then increases with the onset of left-handed supercoiling. The minimum can be used to calculate the number of superhelical turns in the DNA, assuming a value for the angle of unwinding (as defined by the angle between the vectors connecting the glycosidic atoms on two adjacent base pairs) per intercalated drug molecule. Rather than using the stereochemically implausible value of 45° earlier proposed (for proflavine), Waring has used one of 12° deduced from model-building studies of the ethidium-DNA complex [34].

Values of the unwinding angles for drugs other than ethidium can be calculated from their sedimentation behaviour, relative to this (12°) angle.

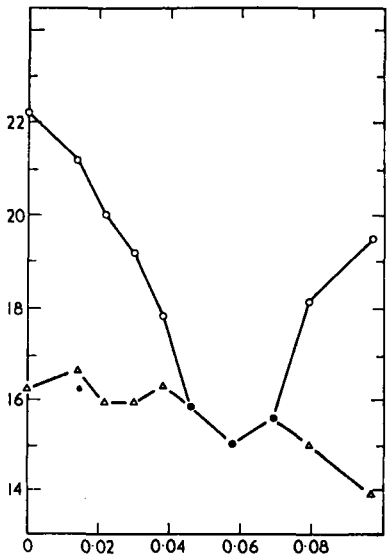
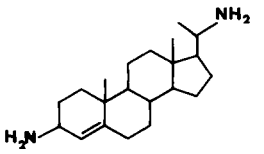


Figure 3.4. Effect of proflavine, expressed as molecules bound per nucleotide, on the sedimentation coefficient of ϕX174 DNA at 20°C [33].

That for proflavine is 8.4°. However, recent careful measurements of ethidium-DNA unwinding in caesium chloride density gradients have indicated that the (superhelical) unwinding angle for this drug is more probably 26° [35]. A value of 8° for proflavine (again relative to 12° for ethidium) has been found from another superhelical unwinding study [36], but on the basis of a modified intercalation model which proposes that drugs produce winding rather than unwinding [37]. However, the basis for this radically different model, namely a novel interpretation of fluorescence depolarisation data [38], has been disputed [39], and the consensus of opinion remains that unwinding rather than winding is a principal consequence of intercalation.



5

The technique of analysing closed circular DNA unwinding is now established as an important diagnostic tool, in order to ascertain whether or not a given drug actually intercalates. A note of caution is necessary however; the steroidal diamine irediamine (5) clearly does not possess the geometric requirements for an intercalation. However, it does unwind superhelical DNA, by approximately half the ethidium angle [40].

KINETIC STUDIES

The proflavine-DNA interactions are fast, and temperature-jump relaxation methods have been found to be suitable for their analysis [41], which have been restricted to the strong binding (process 1, the intercalative region [21]). The observation of two distinct relaxation times indicates two types of complex at equilibrium, and that therefore the binding is two-stage. This has been interpreted as initial fast external binding (*i.e.* the electrostatic type previously considered as process 2), followed by intercalation.

$$\text{Pr} + \text{DNA} \rightleftharpoons (\text{Pr}—\text{DNA})\ \text{OUT} \rightleftharpoons (\text{Pr}—\text{DNA})\ \text{IN}$$

The proportion of externally-bound drug at equilibrium increases with lowering of the salt concentration, whereas moderate changes in pH have relatively little effect. It is also concluded from thermodynamic arguments that the external binding occurs in the major groove of DNA. A stopped-flow kinetic analysis of acridine orange binding to DNA [42] has produced similar results, except that its rate of intercalation has been found to increase with salt concentration. The source of this difference remains obscure.

SEQUENCE AND BASE PREFERENCES IN BINDING

There have been a number of studies directed to determining whether simple acridines bind preferentially to A·T[adenosine—thymidine] rather than G·C [guanosine—cytidine] regions of DNA, in the expectation perhaps that the electronic (and possibly steric) differences between the two types of base pair would be reflected in differing intercalative affinities. A fluorescence quenching analysis of acriflavine binding to DNAs with varying base composition seemed to show that the binding constant increased somewhat with increasing A + T content [43]. However, a subsequent reinterpretation of the data established no such effect [44]. The fluorescence of proflavine-DNA compared with that of proflavine-poly-nucleotide complexes (poly(G·C); poly(A·T); poly(I·C)) (double-stranded polynucleotides with one type of nucleoside only on a strand) has been taken to indicate that intercalation favours A·T sites [45].

A viscometric and temperature-jump study [46] has tended to confirm these results. The increase in length of the DNA molecule on intercalation was greater for A·T-rich DNA (from *Clostridium perfringens* with 70% A·T) than for a more G·C-rich one (from *Micrococcus lysodeikticus*, with 72% G·C). Significant differences were also observed in the kinetic analysis, with only one relaxation time being observed with the latter, in contrast to two for calf thymus DNA [41]. It was concluded that proflavine binds externally to a greater extent with G·C regions than with A·T ones, although Scatchard plot analysis of the binding isotherms suggests a largely intercalative binding for both types of region. A more recent temperature-jump kinetic analysis [47], again using DNAs of varying base compositions has tended to confirm this observation of a difference in bound proflavine forms between the two classes of base-pair regions. In this instance, the kinetic parameters determined, which are comparable to those obtained earlier [41], also indicate that the externally-bound proflavine-DNA complex is more stabilised in G·C regions. It is also suggested that this provides an explanation for the fluorescence quenching of proflavine by G·C, and not A·T, polynucleotides [43—45].

That rather greater specificity than purely base-pair type is possible, has been shown by a NMR and UV absorbance study of proflavine binding to defined-sequence tetranucleotides [48]. Binding is far greater to the duplexes d(CpCpGpG) which is 2′-deoxycytidylyl-(3′,5′)-2′-deoxycytidylyl-(3′,5′)-2′-deoxyguanylyl-(3′,5′)-2′-deoxyguanosine and d(CpGpCpG) than to d(GpGpCpC), establishing an intercalation specificity for pyrimidine (3′,5′)-purine sites—the first two oligomers have one and two such sites respectively, whereas the last has none.

A detailed NMR study of the melting transition of the proflavine-poly(dA·dT)complex has provided direct evidence for intercalation in-between base pairs [49], as well as indications of conformational changes in various regions of the polymer.

BINDING TO POLYNUCLEOTIDES

The biological significance of acridine drug-binding to the various RNAs remains unclear. However, this has not inhibited *in vitro* studies of these interactions, many of which have been prompted by the long-known observation that acridine orange cell staining produces different effects with RNA compared with DNA, which are most manifest in the disparate fluorescence properties of the two types of complexes [23]. The RNA one has a red fluorescence, whereas the DNA one is green. Pronounced differences in the induced circular dichroism spectra of the two complexes has been found

[50], possibly attributable to the difference in conformation between RNA and DNA.

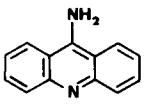
The binding of proflavine to synthetic polynucleotides [51] has indicated a stronger binding to poly(A·U) than to poly(G·C), although qualitatively the interactions are similar to that with DNA, a conclusion reinforced by detailed kinetic and hydrodynamic studies of the proflavine: poly(A·U) complex [52]. Thus, the outside-bound complex is considered to be important, even in the strong binding region. There is also agreement with a kinetic study of the proflavine: poly(I·C) system, which finds rate constants about three times larger than for poly(A·U) [53], with the former polynucleotide binding less strongly.

Transfer RNA has, not surprisingly, been found to bind proflavine [54,55]. It is considered [54] that the binding resembles that to DNA; both intercalative and external modes are involved. A kinetic investigation of the interaction between the single-stranded polynucleotide poly(A), and proflavine and acridine orange [56] suggests that stacking of drug molecules along the polymer chain is involved. (Intercalation in the Lerman sense is, of course, impossible). It has been suggested though [54] that the poly(A):proflavine system exhibits many of the drug's binding characteristics to DNA.

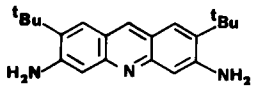
OTHER ACRIDINES

In view of the long-standing interest in their biological properties, it is not surprising that many acridines have been synthesised and examined [13] with a view to improving the efficacy of particular acridine-based drugs. Relatively few though have been examined from the point of view of DNA-binding properties.

In general, substitution of various groups at the 9-position of the acridine nucleus, results in compounds which still intercalate. Thus, 9-aminoacridine (6) binds strongly to DNA by such a process [57,58], although the detailed data obtained indicates differences between the behaviour of this compound (and others more highly substituted at the 9-position) and that of proflavine. A possible interpretation of this may be that the acridine ring nucleus in



6



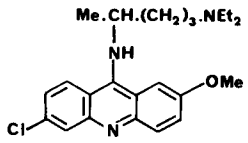
7

proflavine is constrained to lie in a particular position in the intercalation site, because of specific hydrogen bonds from the 3,6-amino groups — no such constraints exists for the purely 9-substituted acridines. Even a bulky substituent at C-9 does not hinder binding; the 9-*sec*-butyl-aminoacridines fully intercalate [59].

Both fluorescence studies [60] and equilibrium dialysis measurements [61] have shown that the introduction of bulky substituents at the 3,6-positions in particular have an adverse effect on binding. It is considered that electronic as well as steric factors are important in this context [60], with preservation of the overall cationic nature of the drug being particularly significant.

The bulky 2,7-di-*t*-butylproflavine (7) has been shown [62] by circular dichroism spectroscopy not to alter DNA conformation on binding, which may be interpreted as indicating non-intercalative binding. This conclusion is reinforced by an extensive analysis [63] of the complex using a number of physico-chemical techniques. Zero unwinding was observed with closed circular DNA. It appears that the externally-bound drug molecule requires a three base-pair site for binding, with two of them being A·T ones. This surprising A·T specificity has suggested that the compound could be of use as a stain (and possibly a selective probe) for A·T-rich regions of DNA.

The anti-malarial drug quinacrine (8) has been clearly shown to be a DNA



8

intercalator [64,65]. Recent spectrophotometric and fluorescence polarisation data have confirmed this [66] and have also shown that quinacrine does not intercalate into poly(I·C) which possesses a double-stranded RNA-11 type structure [8]. The nonetheless strong binding observed with this polynucleotide is presumed to arise from external ionic interactions. This marked preference for the B-DNA structure seems to be a characteristic common to many intercalators possessing an ionisable side chain, which could well participate in binding.

Examination of a series of intercalating acridine analogues with varying hetero-atoms in the central ring [67,68] has shown a relationship between G·C specificity and polarisability of the ring system.

BIOLOGICAL PROPERTIES

Many aminoacridines have long been known to have anti-bacterial activity, and this property has been extensively employed in clinical use for over fifty years (see [13] for a comprehensive account of this topic). Both proflavine itself and acriflavine (a 2:1 mixture of 3,6-diamino-*N*-methylacridinium chloride and 3,6-diaminoacridine dihydrochloride) still find some use in minor wound therapy. Structure-activity studies have shown that anti-bacterial activity is dependent upon the acridine nucleus (or substituents) bearing a net positive charge as well as it having a net planar area of about 38 $Å^2$. Those acridines possessing a 9-alkylamino side chain sometimes display anti-malarial properties, the most prominent and useful example being quinacrine (8) (also known as atebrin or mepacrine). As with the simpler acridines, binding to DNA is believed to be involved in the drug's mode of action [64,65,69].

Binding to DNA is also manifested by proflavine inhibition of both DNA and RNA synthesis using RNA and DNA polymerases [70], the extent of inhibition being independent of enzyme concentration. Quinacrine produces similar inhibitory effects [64,65]. A study of the inhibition of protein synthesis by proflavine in a subcellular rat liver system, as well as in intact rabbit reticulocytes, has been interpreted, on the other hand, as indicating that the drug forms complexes to transfer RNA [55]. This would then inhibit the attachment of amino acids to tRNA. Such a suggestion is not unreasonable in view of proflavine's established *in vitro* binding to this macromolecule; indeed, as with all the drugs discussed in this review, it would be most surprising if there were not other biological receptors besides DNA which assumed some importance in certain circumstances. The related suggestion has been made that RNAs may be the major targets for several chemical carcinogens [71] (whose mode of binding to nucleic acids most probably involves intercalation as well as covalent attachement).

Undoubtedly one of the most intensively studied biological properties of acridines is their capacity to induce frameshift mutagenesis [72—74]. Such mutations to the genetic code add or remove one or several base pairs from the code. This results in the frame of reference having an incorrect starting point for the reading of the code, and so the amino acids eventually produced are not the desired ones. Analysis of such mutations produced in T4 bacteriophage by proflavine led directly to the derivation of the triplet nature of the code [75,76].

A number of molecular models for frameshift mutagenesis have been suggested. The early hypothesis [19] that intercalation on one stand only

during DNA replication was responsible has been discounted for example, by the observation of a lack of correlation between mutagenic activity and replication [77]. Lerman has pointed out [17] that extensive recombination is a characteristic of phages such as T4 which are prone to mutagenic attack by intercalators. He then suggested that whenever there is a drug intercalated at a point on one of the two recombining chromosomal fragments, subsequent pairing will be shifted by a step (*i.e.* 3.4 Å) — this hypothesis can then be extended to explain more than one step being out of register. There are several objections to this model [1], perhaps the most serious being the observation that recombination is not a necessary prerequisite for mutagenesis.

An alternative model for frameshift mutagenesis which has received widespread acceptance, is due to Streisinger, (*Figure 3.5*) [78]. This is based on the ability of acridines to stabilise regions of DNA, rather than that of being able to stretch them by merely an intercalative mechanism of the Lerman type. The model has as its basis the presence of a strand break in the DNA molecule; such breaks can be produced in a variety of circumstances such as misrepair or replication. This is then followed by local mispairing of bases *via* the formation of looped-out regions. These would then be stabilised by the mutagenic drug [74], presumably by stacking. (In the original model, the mispaired region itself was suggested as being stabilised by intercalation). In general, this model has been remarkably successful in many of its predictions, such as that mutagenic events are most likely to occur in regions of monotonous base-sequence and redundancy [73]. However, a serious deficiency of the Streisinger model is considered to be its inability to correlate mutagenic activity with DNA-binding (or indeed intercalative) ability; the model appears to suggest that all compounds with these properties could induce frameshift mutagenesis. This is not the case [73]; it is clear that, in order to understand the phenomenon in detail, it is necessary to take account of individual drug's molecular behaviour and structure; although intercalation in particular is widely discussed in terms of a common mechanism for all such compounds, this principle is unlikely to be correct.

It is known that certain intercalating drugs cause mutations in particular types of base sequence, with runs of G·C base pairs frequently being the affected regions (for example, in [79,80]). A further model for frameshift mutagenesis has been proposed [81], which has as its basis the markedly different fluorescent properties of A·T and G·C base pairs. The latter show a very strong quenching of fluorescence, compared with the fluorescence enhancement of the former, and this model seeks to establish a correlation between these observations and frameshift mutagenicity. Acridine orange, alone among the acridines studied in this analysis, does not show quenching

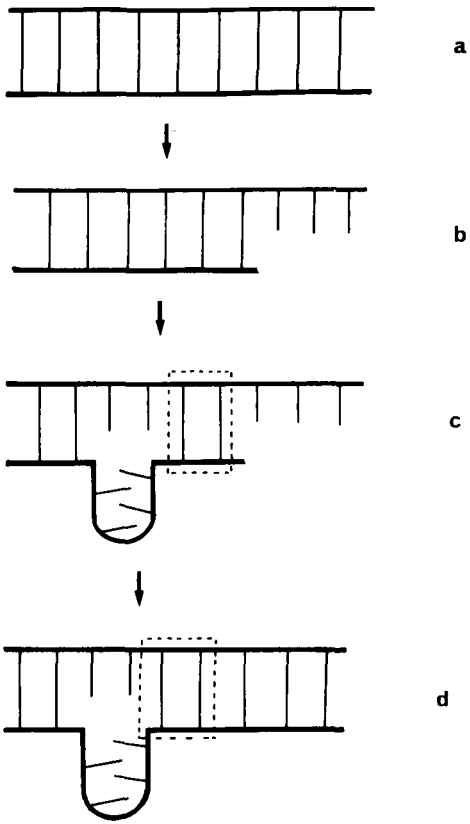


Figure 3.5. The Streisinger model for frameshift mutation (adapted from [78]), showing a possible sequence of events, from (a), a stretch of DNA double helix, with vertical lines representing base pairs. (b) shows strand breakage, from say nuclease attack; in (c) strand slippage has occurred, producing a looped-out region, and mis-pairing (shown within the dotted lines). After subsequent synthesis and/or repair, situation (d) is obtained.

and is not mutagenic for T4 phage (although it is known to be so in the *Salmonella*-microsome test) [82,83]. This model suggests that only a G·C pair can initiate a frameshift, and from this premise a set of rules has been deduced governing the onset of base addition and deletion. Clearly, the inference is that G·C base pairs are involved with acridine intercalation in a particular way; this may be related either to the externally-bound drug complex being stabilised for G·C base pairs, or to geometric or electronic differences from A·T ones.

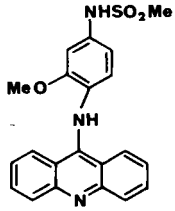
The relationships between mutagenesis and carcinogenesis have been the subject of much speculation, extending over many years. The somatic mutation theory of cancer implies that chemical carcinogens (and other agents such as radiation) produce disease through direct damage to DNA [84]. It is becoming increasingly apparent that the ubiquitous nature of many carcinogens (especially environmental ones) is responsible for the overwhelming majority of human cancers. Since almost all carcinogens are mutagens [82], it has been proposed that a system capable of rapidly testing for this latter property, in preference to the time consuming and costly methods of animal testing for cancer, would offer an invaluable method of screening large numbers of compounds. The so-called 'Ames test' [82,83] employs strains of the bacterium *Salmonella typhimurium*, together with a preparation of liver homogenate microsomal enzymes (which often metabolises otherwise inactive compounds to active forms); in this way a fair representation of mammalian metabolism is obtained. *Table 3.1* shows results obtained for a number of acridines and other DNA-binding drugs. It is clear that such compounds both present considerable hazards (a subject which shall be returned to later in this review) and also potentially provide us with powerful tools for probing the carinogenic process.

It is perhaps not surprising that several acridine derivatives are currently being developed as anti-cancer agents. Thus, 9-(3-dimethylaminopropylamino-1-nitroacridine) (1-nitro-9-(3′-dimethylamino-*n*-propylamino)acridine) has been reported to possess useful anti-tumour activity [85]. It has been found that this compound binds to the DNA of Ehrlich ascites tumour cells *in vivo*; presumably both covalent and intercalative mechanisms

Table 3.1. THE MUTAGENIC AND CARCINOGENIC ACTIVITY OF SOME INTERCALATING COMPOUNDS [82]

Compound	*Carcino-genicity*	*Muta-genicity*	*Revertants per nmol*
9-Aminoacridine	?	+	10
Acridine orange	+	+	66
Proflavine	?	+	38
Ethidium bromide	?	+	80
Adriamycin	+	+	108
Daunomycin	+	+	356
Hycanthone methanesulphonate	+	+	2.5

The number of revertants per nanomole is considered to be a rough guide to mutagenic potency in the *Salmonella* test.



9

are involved. The acridinylmethanesulphonanilide family of compounds [86], of which *N*-[4-(9-acridinylamino)methanesulphon-m-anisidide,9) is a prominent example, are believed to act by binding to DNA via an intercalative process. Compound (9) removes and reverses the supercoiling of closed circular PM2 DNA [87], with an estimated unwinding angle of about 20°, relative to one of 26° for ethidium. The compound shows pronounced activity against L1210 leukemia and other experimental cell systems. However, the *o*-anisidide derivative, although it unwinds PM2 DNA to almost the same extent, is devoid of activity. Clearly, factors other than just DNA affinity are of importance in determining activity for this class of compound.

OTHER DNA-BINDING DRUGS

ETHIDIUM BROMIDE

Ethidium bromide (2) is a phenanthridine analogue which has found widespread use in the treatment of trypanosomiasis [88]. It is also active against the growth of bacteria, viruses and mammalian cells. The drug induces ultrastructural changes which resemble those produced by acridine drugs. It is considered that the selective toxicity of ethidium to trypanosomes arises from differential membrane permeability properties [88]. Nucleic acid synthesis, both *in vivo* [89] and *in vitro* [90], is strongly inhibited. There is considerable evidence that the *in vivo* target of attack is mitochondrial (*i.e.* extra-chromosomal) DNA, which exists largely in a closed circular form [88,91,92] in most, if not all, organisms. Ethidium bromide is extensively employed in molecular biology as a stain for nucleic acids and chromatin, and as a probe for their structure and function (for example, in [93]).

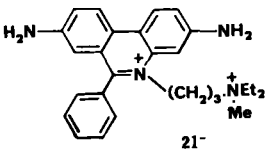
The nucleic acid synthesis-inhibitory properties of ethidium bromide [90] strongly suggest that binding to DNA is responsible for this effect. *In vitro* studies of the drug-DNA interaction have shown similar effects to those produced by proflavine and other acridines; in some instances, the effects are

even more pronounced. The large metachromatic shift, from 480 nm to 520 nm, is a striking example [94]. The binding process has two regions; a strong primary one due to intercalation, which is followed by a weaker secondary stage due to external stacking. The tendency of ethidium to stack is considered to be rather less than for the acridines [94]. The kinetics of the DNA-ethidium strong binding process are complex, involving a number of steps [95]. Evidence for intercalative binding is also revealed by hydrodynamic studies of the complex, which show an increased viscosity and decreased sedimentation coefficient relative to DNA [96], and by the reversal of supercoiling in closed-circular DNA [33]. An NMR study of the shifts in the drug's proton resonances in a complex with poly(dA·dT) has been interpreted as indicating intercalation [97].

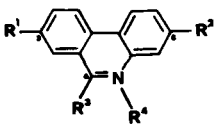
Ethidium binds in a similar manner to double-stranded RNA [98]; single-stranded polynucleotides show no evidence of strong binding. Triple-stranded helices are de-stabilised by the drug [99]. The unwinding angle for intercalation into a poly(A·U) double helix has been determined by a pulse fluorimetric method to be about 38° per base pair [100]. This value is greater than that determined for DNA by the same technique [101] (16°), and (reliably) by alkaline titration in caesium chloride density gradients of superhelical DNA [35] (26°).

Several studies have reported on the binding of ethidium bromide to transfer RNAs. It is considered that, since these relatively small nucleic acids (of average molecular weights about 25000) are amenable to detailed structural analysis, they are hence suitable model templates. A high-resolution proton NMR study of a yeast phenylalanine tRNA-ethidium complex has revealed a unique binding site [102], intercalative in nature, on the base-paired region of the amino-acid acceptor stem. This single-site interpretation has also been suggested for several other tRNAs [103]; at higher drug levels multiple-site non-intercalative binding is observed. Only this latter type was found in the crystal structure of an ethidium-tRNA complex [104], with the drug located in a non-helical fold of the molecule. This discrepancy between the solid-state studies and those in solution may be attributable to the method of preparation of the crystalline complex, which was obtained by soaking crystals of the native tRNA in ethidium bromide solution. The rigid tertiary crystal structure of tRNA is probably not amenable to the alterations necessary to unwind base-paired regions.

Preferential sequence binding has been observed for the interactions of ethidium bromide [105—107] and propidium di-iodide (10) [107] to dinucleoside phosphates. A marked preference for binding to pyrimidine (3′,5′)—purine sequences was found. Evidence for the existence of Watson-



10



11

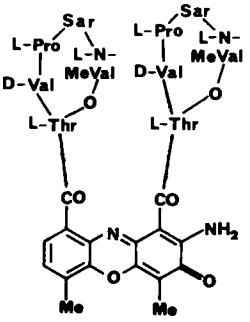
Crick base paired intercalated dimers was obtained for such sequences, particularly with the dinucleoside CpG. The same sequence preference has also been noted at the tetradeoxynucleotide level [108].

The DNA-binding properties of a number of phenanthridinium derivatives (11) have been examined [88,109], many of which had been previously tested for both antibacterial and trypanosomal activity [110]. The presence of amino groups at R^1 and R^2 is an important requirement for maximal binding, unwinding of PM2 DNA, and biological activity. Removal of the phenyl group from the R^3 position leads to reduced unwinding of closed-circular PM2 DNA, as well as a lower binding constant. This type of derivative also has markedly diminished biological activities; it has been suggested [109] that such a molecular has a much more planar geometry than ethidium itself and therefore has a higher tendency to stack on the exterior of the DNA double helix. The nature of the quaternary group R^4 has little effect on either binding or activity, suggesting that this group is some distance from the intercalation site, and possibly projects into a groove of the DNA.

ACTINOMYCIN

The actinomycins are a family of antibiotic metabolites isolated from several *Streptomyces* [111]. All contain a phenoxazone chromophore linked to polypeptide groups. Actinomycin D, the compound most widely studied biologically in the series, has two identical cyclic pentapetide groups (12). It is a very potent anti-tumour agent and has found clinical application in the treatment of choriocarcinoma and a few other rare cancers; the extreme toxicity of the drug has precluded it from more general use [112]. Actinomycin D, perhaps more than most intercalating drugs, has often been employed in molecular biology as a probe of nucleic acid functional processes.

Actinomycin D specifically inhibits DNA-directed RNA synthesis in a wide variety of cellular systems [113,114] by binding directly to the DNA template [115]. Binding to double-stranded RNA is minimal. The association constant is about 5×10^6 mol^{-1}. Studies of binding with purine and pyrimidine



12

nucleosides and nucleotides have suggested that the presence of deoxyguanosine is an essential requirement for the binding [116]. Thus, in DNA, a G·C base pair is suggested as the site of binding. Furthermore, since poly(dI)·poly(dC) does not bind the drug at all [117], the 2-amino group of guanine is a requirement for interaction. Several features of the actinomycin molecule are also essential for activity, particularly the amino and quinonoid groups on the chromophore. These factors have been interpreted as involving specific hydrogen bonds in the binding [118]. Hydrodynamic, spectroscopic and kinetic data for the actinomycin-DNA complex [115] are believed to favour intercalative drug binding, a conclusion given support by the finding that the drug removes and then reverses the supercoiling of closed circular DNA [33], with about the same unwinding angle as ethidium.

A number of molecular models have been suggested for the actinomycin-DNA complete. The first [118] involves hydrogen bonds between a guanine base and the chromophore, with both this and the cyclic peptides (themselves hydrogen-bonding to phosphate oxygen atoms) tightly packed into the DNA small groove. The chromophore is inclined at about 70° to the helix axis. On the basis of data strongly supporting intercalation of the chromophore, a model incorporating this feature has been proposed [115], which, although satisfactory in many respects, fails to take account of the guanine specificity. A second non-intercalation model [119] involves helical unwinding, so that the actinomycin molecule, again in the narrow groove of the DNA, spans about six base pairs (rather than three [118]), in good agreement with the binding data.

The most recent model has been proposed on the basis of the crystal structure of a 1 :2 actinomycin :deoxyguanosine complex [120,121] and has commanded widespread acceptance. This structure is also of some significance in being the first direct atomic-level visualisation of a drug bound to (a model

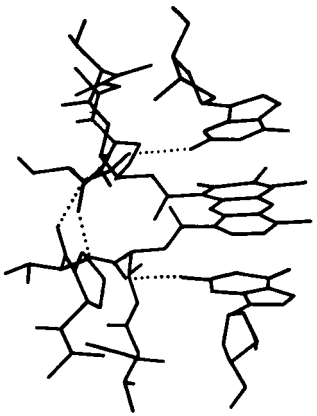


Figure 3.6. The crystal structure of the actinomycin-deoxyguanosine complex viewed from a sidewise direction. Dashed lines indicate hydrogen bonds. (Redrawn from [120]).

for) its receptor. The principle features of the nucleoside complex (*Figure 3.6*) are its almost exact two-fold symmetry with the guanine bases stacking on either side of the chromophore. Strong hydrogen bonds connects the guanine 2-amino group with the carbonyl oxygen atom on the L-threonine residue of actinomycin, thus providing an explanation for the guanine specificity of the drug. The model proposed for the complex with DNA [120,122,123] incorporates many of these observations. The area of binding in the complex has two-fold symmetry and has the phenoxazone group intercalated in between two dG·dC base pairs. The self-complementary G·C sequence thus maximises the guanine-threonine hydrogen-bonding requirement; support for this comes from the studies on polynucleotide complexes [117], as well as NMR studies on complexes with pd(GpC) complexes [124—126] and with the hexanucleotide d(ApTpGpCpApT) [127]. This sequence is that proposed for the model of the actinomycin-DNA complex [120,122,123], which was constructed (by molecular model-building) by adding appropriate residues to either side of the dG—dC duplex. The unwinding of these base pairs was estimated to be 18°, with additional unwinding of the two proximal A·T base pairs, of 8°. Thus, the total unwinding induced by actinomycin was considered to be $(18 + 2 \times 8)$ *i.e.* 34°. The model also has the pentapeptide groups lying in the narrow groove of the double helix, stabilised by hydrogen bonds and spanning about six base pairs.

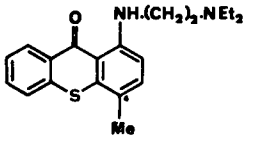
The model has also enabled explanations to be given concerning the biological activity of several actinomycin D derivatives [123] — for example,

when L-proline in one of the pentapeptides is replaced by a sterically bulkier group (which would not fit so well into the narrow groove), activity is reduced up to sixty-fold (in actinomycin $X_0\beta$Ac). The two-fold symmetry visualised in this complex has been utilised in attempts to understand nucleic acid—protein recognition processes [128,129].

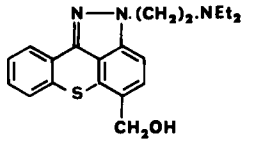
A detailed circular dichroism study has been made of actinomycin binding to a number of both natural and synthetic DNAs, in which nearest-neighbour frequencies were analysed [130,131]. It is concluded that both GpC and CpG sites are favoured for the drug, casting a measure of doubt on the above model, which only allows the former one.

MIRACIL D

The synthetic compound Miracil D (lucanthone) (13) was developed as part of a systematic search for compounds with potential schistosomicidal activity [132]. Subsequent work established the 4-hydroxy methyl derivative, hycanthone, to be a major metabolite [133]; it has been found to have greater therapeutic activity than the parent compound. Both Miracil D and hycanthone have been of major clinical importance in the treatment of schistosomiasis. In recent years, the finding that these compounds are highly mutagenic in several bacterial test systems [134], has called into question the wisdom of using these drugs on humans. Hycanthone in particular is a potent frameshift mutagen for *Salmonella* [134,135]. However, various indazole derivatives (for example, (14)), showed no appreciable mutagenic effects when



13



14

tested in *Salmonella* and bacteriophage T4 systems; malignant transformations also failed to appear in cells infected with Rauscher virus [136]. Moreover these Miracil analogues had equal or even greater antischistosomal activity than the parent compounds. This clear separation of schistosomicidal from mutagenic properties indicates that they operate by quite distinct biochemical mechanisms. In spite of extensive investigation, remarkably little

is known about the precise mechanisms of the former property of Miracil D and its cogeners [132], although many suggestions have been put forward (such as inhibition of mitosis or motility, or interference with carbohydrate metabolism). In contrast, the mutagenic properties of these compounds in bacterial systems are relatively well understood. This is perhaps not too surprising when one compares the great complexity of the schistosome parasite and its life-cycle, with that of many, well studied bacterium (such as *E. Coli*).

Both Miracil D and hycanthone are effective against a wide range of laboratory tumour cell lines [132], with the latter active in virtually every tumour system tested [132,137]. As yet, unacceptable toxicity has precluded their use in clinical treatment. However, Miracil D, like actinomycin D, enhances X-ray damage in mammalian cells, a property of potential use in anti-tumour radiotherapy [138].

Miracil D has a profound effect on bacterial growth, causing complete inhibition of RNA synthesis in *B. subtilis* and *E. Coli* [139,140]. Inhibition of DNA synthesis is highly dependent on the cell system, being pronounced for mouse leukemia L1210, and absent for *B. subtilis*. These and other results strongly suggest that Miracil D acts by directly binding to DNA [141].

The absorption maxima are shifted to longer wavelengths, by about 7 nm, on interacting the drug with DNA; synthetic polynucleotides produce rather smaller effects, with poly(A) having the largest within this group. The drug also produces an increase in the relative viscosity of DNA [142]; this property is abolished when the ionic strength is high. Similarly, the thermal denaturation profile of DNA is altered on binding of Miracil D [141,143], with an increase in melting temperature (T_m) of up to 23°C being found. All this evidence is highly suggestive of an intercalative mode of binding, which is reinforced by the observation that the drug removes and reverses supercoiling in closed circular DNA [33], with an unwinding angle of 6.8° for hycanthone (again relative to one of 12° for ethidium). In view of the structural requirement for an intercalative drug being that the ring system be planar, X-ray crystallographic analyses of both Miracil D [144] and hycanthone, as the methanesulphonate salt [145], have been reported. Whereas the antipsychotic thioxanthene drugs, such as flupenthixol [146], have a folded (130—150°) conformation about the central carbon—sulphur axis, that of hycanthone is appreciably flatter, with a 168° angle, and Miracil D itself has the chromophore almost completely flat (*Figure 3.7*). The structures of derivatives such as (14) are reported also to have planar chromophores [145], reflecting the severer steric constraints to non-coplanarity in these compounds. The cationic charge resides on the terminal chain nitrogen atoms [145].

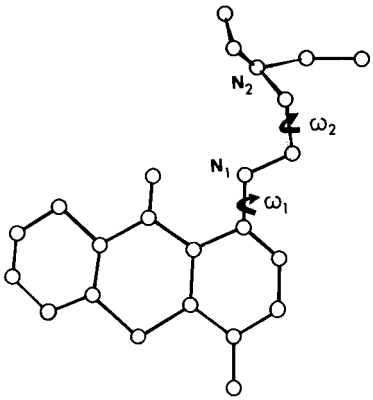


Figure 3.7. The structure of the Miracil D molecule, as found in the crystal structure [144].

The interaction of Miracil D with poly(A·U) having 20—25 nucleotides in the chain has been examined by proton NMR spectroscopy [147]. It is concluded that the drug indeed intercalates in-between base pairs, with the side-chain extending out into the major groove so that the terminal nitrogen atom hydrogen-bonds to a phosphate oxygen atom at the intercalation site. This role for the side-chain in binding has been previously suggested [141] on purely steric grounds. The side chain is free to adopt any one of a number of conformations (*Figure 3.7*); thus in Miracil D [144], the torsional angle ω_2 is 77°, whereas in hycanthone [145], it has a value closer to 180°.

The DNA-binding properties of a number of Miracil D derivatives have been examined in several studies. The effects of four indazole analogues of type (14) on the sedimentation properties of closed circular PM2 DNA have been investigated [148], all of which bind to DNA and induce an unwinding angle, which for three of the compounds, is close to that found for hycanthone itself [33]. The fourth derivative, which has a chlorine atom attached to the chromophore, has an unwinding angle about a third lower than the others; it is of interest, though of obscure significance, that this was the one derivative totally non-mutagenic in all tests. An examination of Miracil D analogues with varying side-chain substituents [143] has been made using three test methods; DNA thermal denaturation, inhibition of *Bacillus subtilis* growth, and inhibition of RNA polymerase activity. In almost all cases, there was significant correlation between the methods; thus, a low ΔT_m value, indicating little additional helical stabilisation, was accompanied by little growth-inhibitory activity. The essential molecular requirements for binding

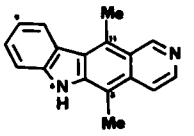
and activity were deduced to be: a non-alkyl-substituted proximal nitrogen atom, and the presence of a (specially) correctly-positioned terminal nitrogen atom. This latter is in accordance with the steric requirements of the DNA-binding model discussed above. An exception though, to the proximal nitrogen having to be (by implication), protonated, was found in the 6-chloro series. It has been suggested that this implies a rather different mode of DNA binding [141—149], perhaps involving an alternative position of the chromophore inbetween the intercalated base pairs.

The crystal structures of Miracil D and hycanthone [144,145] established that the torsional angle ω_1 (*Figure 3.7*) was close to zero degrees. This established the existence of an intramolecular hydrogen bond, previously postulated from spectroscopic data [150], between the proton attached to the proximal nitrogen atom, and the carbonyl oxygen. The hydrogen bond serves to stabilise the conformation in this region of the molecule, which is necessarily destroyed on *N*-alkylation [151], for purely steric reasons — the bulky alkyl group forces the whole side-chain out of co-planarity with the chromophore.

Several recent studies have suggested that Miracil D and hycanthone binding to DNA *in vivo* is rather more complex than the process of intercalation presented here so far, suggests [152,153]. The former compound produces strand breaks in DNA from HeLa cells, whereas hycanthone does not. Their mutagenic activities differ considerably, with hycanthone being a much more powerful mutagen. *In vitro* DNA-binding differences between the two drugs have been known for some time [142], with lucanthone increasing the relative viscosity of DNA to a much greater extent than hycanthone. These observations imply that the two drugs bind to DNA by different intercalative modes; as yet, no detail at all at the molecular level is available which might shed light on this problem. Such information might well be relevant to the differences in mutagenicity, though of course totally different factors might be involved.

ELLIPTICINE

Ellipticine (15) is a plant alkaloid which has pronounced antineoplastic activity in experimental mouse L1210 leukemia and other cell lines [154,155]. This planar compound, which is protonated in mildly acidic conditions, has approximately the same molecular dimensions as proflavine. Thus, in view of its known interaction with DNA [156], it is reasonable to expect intercalation to be involved. A thorough study [157] has established this to be the case. The absorption spectral changes on binding to DNA are typical of intercalators;



15

the apparent association constant of 10.3×10^6 mol^{-1} indicates somewhat stronger binding than proflavine, though the extent of unwinding of PM2 DNA (7.9°) is very close to that for proflavine (8.0°). Data from viscosity and electric dichroism studies also support intercalative binding for ellipticine, with the latter technique showing that the plane of the molecular rings lies parallel to base pairs.

A systematic study has been made of the DNA-binding properties of various ellipticine derivatives [158,159] in an attempt to correlate DNA affinity with pharmacological (especially anti-cancer) activity, and hence rationally develop the most effective possible compound in the series. Both (apparent) binding constants and PM2-DNA unwinding angles have been compared with anti-cancer activity in mouse L1210 leukemia. Since the protonated forms of these compounds are considered to bind more strongly to DNA than the neutral ones, the cationic binding constants were also evaluated (*Table 3.2*). The apparent binding constants were obtained [158] by

Table 3.2. DNA-BINDING PROPERTIES AND EXPERIMENTAL ANTI-CANCER ACTIVITY OF SOME ELLIPTICINE DERIVATIVES [158]

Compound	K_{app}	K_E^+	*Unwinding angle*	*Activity*
6-Isopentylellipticine	$< 10^4$	6.3	8.8	0
5,11-Dimethylellipticine	1.0×10^4	5.1	—	0
11-Methylellipticine	2.4×10^4	5.5	—	0
9-Methoxyellipticine	1.0×10^5	5.7	6.8	90
Ellipticine	1.5×10^5	5.2	9	94
9-Bromoellipticine	4.0×10^5	6.9	0	0
9-Aminoellipticine	1.2×10^6	6.1	4	0
9-Hydroxyellipticine	2.0×10^6	6.1	12	100

K_{app} is the apparent binding constant, (in mol^{-1}) at pH 7.4 (except for the 6—130 penthyl derivative, measured at pH 5.0. Unwinding angles were also measured at these pH values. K_E^+ is the calculated cation binding constant. The anti-cancer activity is expressed as % of L1210 cells killed by a third of the dose killing 50% of the animals.

measuring their ability to compete with ethidium in binding the DNA—possibly explaining the discrepancy by a factor of seven, between the K_{app} for ellipticine itself determined in this study and that by more orthodox methods [157]. The data obtained, even in a qualitative manner, support the conclusion of a positive correlation. The two apparent exceptions to this are both explicable; the 9-bromo derivative is considered to bind by a non-intercalative mechanism whereas the 9-amino compound is probably metabolically inactive (witness its low toxicity). The actual extent of unwinding angles is a poor guide to activity, as one might expect. More surprisingly though, is the report [160] that the anti-tumour properties of these compounds do not correlate at all with their mutagenic properties, as tested in Ames-type *Salmonella* tests. Thus, 9-bromoellipticine is a powerful mutagen whereas the 9-hydroxy derivative is not. These observations are possibly therapeutically significant in that they point to active ellipticine analogues that seem to be devoid of the carcinogenic nature inherent in most anti-cancer drugs.

BIFUNCTIONAL INTERCALATORS

The hypothesis that increasing strength of DNA binding is positively correlated with improvements in therapeutic index (be it for example, as

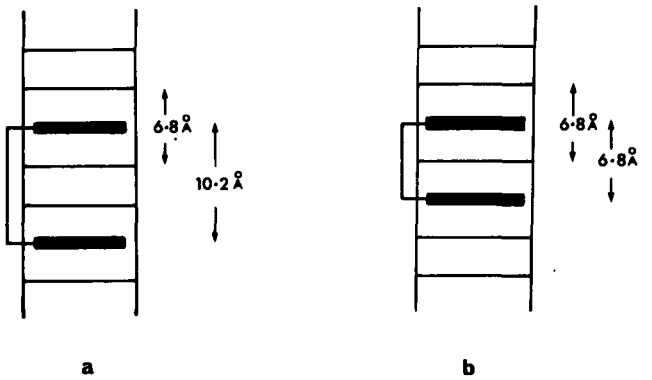
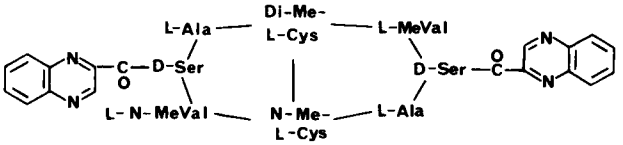


Figure 3.8. Bifunctional intercalation into a DNA double helix, with the drug chromophores shown in solid outline, linked together by a chain of atoms largely outside the helix. The base pairs of the DNA are shown as horizontal lines, connected to the vertical lines as backbone. In (a), the chromophores are sufficiently far apart for two base pairs to be inbetween (neighbour exclusion). There is only one base pair between them in (b).

applied to anti-tumour or anti-bacterial activity), is undoubtedly a simplistic one. Nevertheless, as we have already seen, there are many instances where this theory is broadly correct. Endeavours have been recently been made to capitalise on this by utilising compounds that intercalate simultaneously at more than one base-pair site. Such compounds, with more than one intercalating chromophore, could well have significantly higher binding constants than mono-intercalating drugs. Bis(methidium)spermines (the *N*-methyl analogue of ethidium) has two methidium groups linked by a spermine-molecule chain [161]. Its binding constant for the DNA interaction is at least 4×10^9 mol^{-1}, with one molecular binding to every four base pairs and an unwinding angle about one and a half times that of ethidium itself. These results are in agreement with a bis intercalated species, as well as with the neighbour exclusion principle (*Figure 3.8a*) [1].

Echinomycin

The near-perfect two-fold symmetry of echinomycin (16), with two quinoxaline chromophores linked by an octapeptide groups, suggests that its binding to DNA utilises this property in some way [162]. Binding studies have been hampered by the lack of solubility of the drug in aqueous media; however a solvent-partition method [163] has successfully overcome this problem.



16

Echinomycin binds specifically to DNA and not to RNA, with double-stranded DNA being preferred over the single-stranded form [164,165]; completely denaturing the DNA totally abolishes binding. The apparent binding constant for binding to calf thymus DNA is about 6.0×10^6 mol^{-1}. Generally, G·C-containing polynucleotides have the highest affinity for the drug, although this simple picture is complicated by an indication of sequence effects being of importance. Interactions with synthetic polynucleotides does not seem to correlate with what is known about their structures [8]; for example, poly(dG·dC) binds echinomycin much more strongly than the alternating polymer poly(dG·dC).poly(dG·dC), even though the former has

an A-DNA type structure, closely similar to that of double-stranded RNA (which does not bind the drug). The alternating dG·dC polynucleotide has a B-DNA type structure, which *a priore*, would be expected to bind the drug much more tightly than poly(dG·dC).

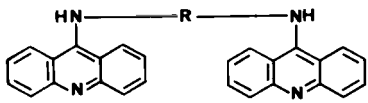
Measurements have been made on the increase in length of sonicated DNA fragments on echinomycin binding [164,165] by means of the increase in viscosity produced in the macromolecule. The helix extension is about 6.3 Å per bound drug molecule, compared with about half this value for proflavine or ethidium. The helix-unwinding angle is also almost double that for ethidium. These results confirm that echinomycin indeed intercalates bifunctionally, with both chromophores slotting in simultaneously (it is of interest that the simple mono analogue, quinoxaline-2-carboxamide, does not intercalate at all [162]).

In the absence of a definitive crystal structure, molecular model-building techniques and semi-empirical potential energy calculations have been used [166] in attempts to define a likely conformation for echinomycin. The most plausible structure has a pseudo two-fold axis of symmetry passing through the central sulphur atoms of the cross-bridge. The quinoxaline chromophores in this model are almost parallel, and are separated by 10.2Å. Thus, they are ideally positioned to take part in bifunctional intercalation (*Figure 3.8a*) since the interplanar gap is (3 × 3.4 Å), which satisfies the minimum condition for the neighbour exclusion principles [1,167] to be obeyed. It is pointed out that this model and a subsequent revision with the chromophores now coplanar with adjacent peptide linkages, which agrees better with circular dichroism and NMR data [168], must await verification by X-ray crystallography.

However, the binding data for the echinomycin-alternating polydeoxynucleotide systems [162—164] suggests a serious conflict with the neighbour exclusion principle. Whereas the number of base pairs occupied by a drug molecule was found to be at least four for various DNAs and poly(dG·dC), that for the alternating copolymers was three. This necessitates only one base pair between adjacent intercalated echinomycin molecules, clearly violating the principle. Further data are needed before firm conclusions can be drawn, especially a confirmation that bifunctional intercalation actually takes place in these polymers.

Diacridines

Acridine chromophores have been used in a number of investigations, as the repeating unit in synthetic bis-intercalators (17). The linking units (R) employed have been simple aliphatic chains, of easily alterable chain length.



17

The crystal structures of several such compounds have been solved [169,170] and show the chains to have a fully extended conformation.

Compounds with R = $-(CH_2)_3-NH-(CH_2)_4-(CH_2)_3-$ and $-(CH_2)_3-NH(CH_2)_4-$unwind PM2 DNA by 38°[171], whereas both the analogous monoacridine and the diacridine with R = $-CH_2)_3-NH-(CH_2)_3-$produce unwinding of 17°. Apparent binding constants for the first two were found to be several thousand times larger than for the monomer. It is concluded that these two compounds bis-intercalate, whereas the third dimer only mono-intercalates; a minimum separation of 10.2 Å between chromophores is required for bis-intercalation since the third diacridine has a maximum chain length of 9.9 Å. An NMR study has been made of the interactions between (17), with R = $-(CH_2)_3-NH(CH_2)_4-NH-(CH_2)_3$(spermine diacridine) and 3′-AMP, 5′-AMP and ApA respectively [172]. The data was interpreted as suggesting that the two mononucleotides have their bases intercalated in-between the two acridine chromophores, whereas the ApA dinucleoside forms two completed species.

The polynucleotide-binding and biological properties of a number of diacridines have been examined [173—175], both for spermine-type analogues and for those with R = $-(CH_2)_n-$(n = 4—18). Spermine diacridine greatly stabilises the DNA double helix, as measured by a ΔT_m of 44° (in low salt compared with 11°C for 9-aminoacridine). Variation of ΔT_m with increasing chain length shows a maximum with n = 12. The inhibition of P-388 leukemia cell growth in culture correlates with the increase in ΔT_m; however p-388 tumours *in vivo* showed optimum tumour growth inhibition at n = 6, due presumably to increasing host toxicity after this point. Both biological effects show a considerable enhancement compared with the parent monoacridine. The primary site of action of these compounds has been suggested to be inhibition of RNA synthesis [173], in accord with the supposition of their binding to DNA *in vivo*. Electron microscopy of HeLa cells after exposure to spermine diacridine [175] provides supporting evidence for this. Further data have shown that both chain initiation and elongation are inhibited by the compound.

However, a more recent study of the *in vivo* tumour activity of a number of diacridines has suggested a rather different picture [176]. Attempts were made

to correlate the percentage increase in life-span of P-388 leukemia cells in mice when drug was administered, with (a) extent of inhibition of RNA or (b) DNA synthesis, (c) the extent of uptake of drug by P-388 cells, or (d) the rat of S-180 cell agglutination, for a large number of differently-substituted diacridines. Only in the case of (d) was any correlation perceived; this was a highly significant inverse one. The mono-intercalating diacridines ((17) with $R = -(CH_2)_2-$ or $-(CH_2)_4-$), did not fit the correlation. These experiments were indicative of interactions with specific membrane sites, although cell-growth inhibitory properties (*i.e.* DNA binding) were considered to be of importance for the full expression of antitumour activity.

The ability of the diacridines with $R = -(CH_2)_n-$ to unwind closed-circular PM2 DNA, has been thoroughly investigated [162,177]. The transformation from mono- to bis-intercalation was found to occur between $n = 4$ and $n = 6$, with the latter compound clearly acting bifunctionally whilst the $n = 5$ one gave 'intermediate' results dependent on the level of binding. The clear finding of bifunctionally for $n = 6$ is of considerable interest; the $-(CH_2)_6-$ chain can have a maximum length of only 8.8 Å which necessarily means that the binding must be of the type shown in *Figure 3.8b*. The neighbour exclusion model of Figure 8A is then ruled out. (The apparent variance between this result and that obtained from other diacridines [171] may be explicable in terms of the differing nature of the inter-acridine chains). In contrast to their behaviour with DNA, the diacridines do not appear to bis-intercalate into the RNA analogue poly(A·U) [178]—this finding will be discussed further in the following sections.

MOLECULAR MODELS OF DRUG—DNA BINDING

The concept of intercalation, as originally defined [15—19], has been most useful in providing an insight in the most general terms into the DNA-binding properties of the various drugs outlined in the preceding sections. However, the model proposed (*Figure 3.3*) lacks that stereochemical detail necessary for a more complete understanding of these processes. Accordingly, physical methods of structure determination have been employed in attempts to provide a more complete model for intercalation. Up to the present time, these have been almost completely dominated by X-ray diffraction methods — the example of the protein field has shown that these are unsurpassed in the wealth of molecular detail that they can provide. However, nucleic acids (except for transfer RNA) have far too high a molecular weight for single-crystal methods to be employed. Hence, the structural data obtained to date have been from

two somewhat indirect methods, neither of which has as yet provided the definitive information sought. Indeed, it can be argued that recent results, in showing the hitherto unsuspected structural complexity of the intercalation process, have pushed this goal further into the future.

FIBRE-DIFFRACTION STUDIES

The structures of both DNA and RNA, as well as those of many polynucleotides, have been established by this method [8], which involves analysing the diffraction pattern obtained from a sample of the polymer pulled into the form of a fibre. The structure has to be deduced on the basis of the best fit to an assumed molecular model (although procedures are now available [179] to refine this model against the X-ray data to obtain a computerised best-fit, with the conformation of the model being subject to some alteration in the process). In addition, for the natural polymers, subtle details of structure due to sequence-dependence are lost since the X-ray data obtained represent diffraction averaged over the whole polynucleotide chain. Thus, the model finally obtained is itself an averaged one [180]. Finally, fibre diffraction data do not extend to atomic resolution; models obtained from them implicitly reflect this feature.

Fibres of drug-DNA complexes invariably produce diffraction patterns that have considerably less detail than those produced by the polymer alone, due to molecular disorder within the fibre. The disorder can most likely be ascribed to the effects of non-random intercalation, and significant external binding [181]. This has meant that intercalation models based on such studies have considerable geometric latitude. Analyses of drug-polynucleotide complexes, (especially of the synthetic DNAs with repeating di- and trinucleotide sequences that are now available), could well provide higher-quality fibre diffraction data and hence better models. As yet no studies on such systems have been reported.

Mention has already been made of the original X-ray analyses of DNA-proflavine fibres. An unwinding of 45° for the base pairs at the intercalation site was first proposed [15]. This was subsequently modified to one of 36°, leaving the base pairs exactly parallel (B-DNA, with ten base pairs per turn, has an angle of 36° between adjacent base pairs).

The molecular model for the ethidium-DNA complex [34] has the drug intercalating from the large groove of the polymer, leaving its bulky phenyl and ethyl groups protruding into this space. The complex is stabilised by hydrophobic stacking forces and also possibly by hydrogen bonds between phosphate oxygen atoms and the drug's amino groups. The unwinding angle

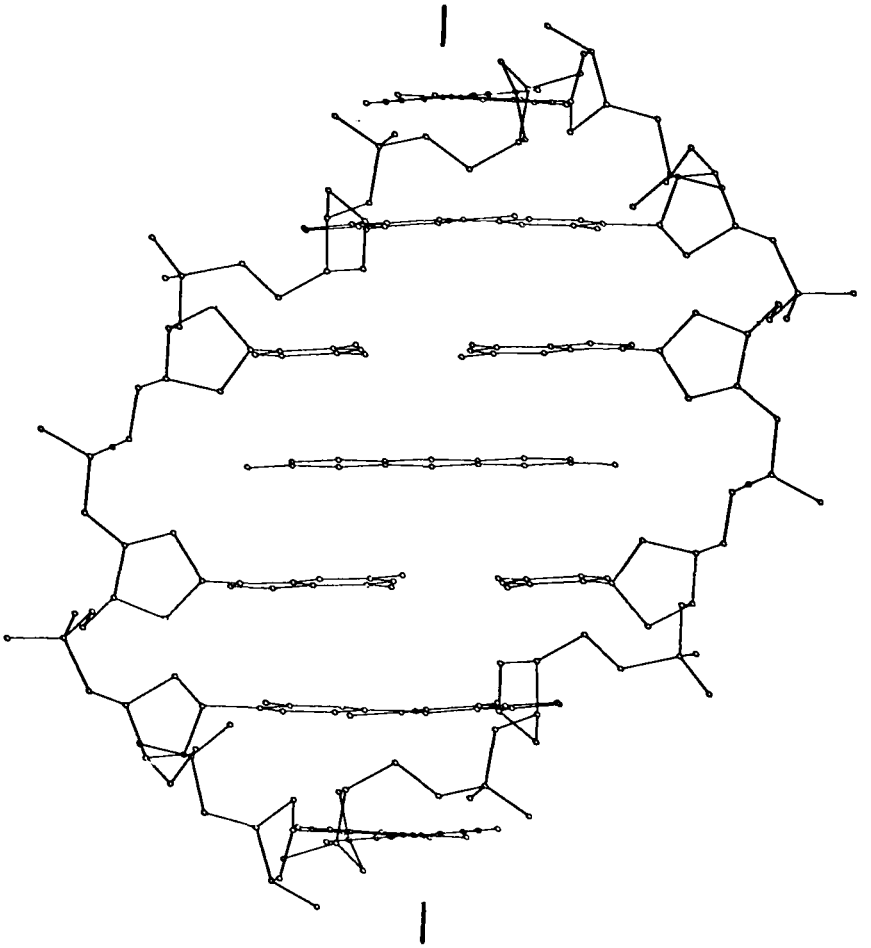


Figure 3.9. The optimum computed stereochemistry for the B-DNA-proflavine complex [182]. The vertical lines represent the axis of the polynucleotide double helix. The view is from the minor DNA groove.

was considered to be around 12° for steric reasons. This was thought to be a minimum value, although lower ones have been subsequently found relative to this, from closed-circular DNA binding studies [33]. This anomaly might [1] reflect real structural differences between intercalative drugs or may be possibly be due to interference from externally-bound drug molecules. A

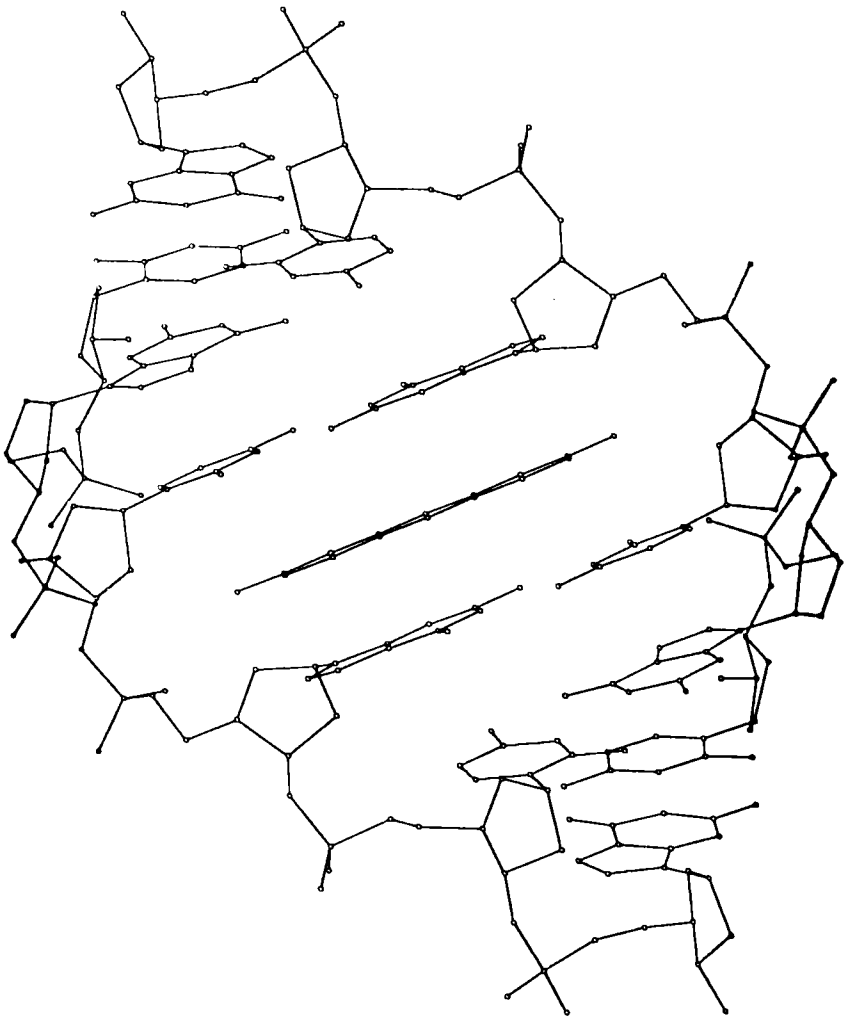


Figure 3.10. The computed optimum configuration for an A DNA-proflavine complex, viewed facing the major groove [183].

diffraction analysis of proflavine and acridine orange-DNA fibres [181] has tended to confirm the existence of such non-intercalated forms concurrently with intercalated ones. This study also suggests that an unwinding angle of around 12° is most likely.

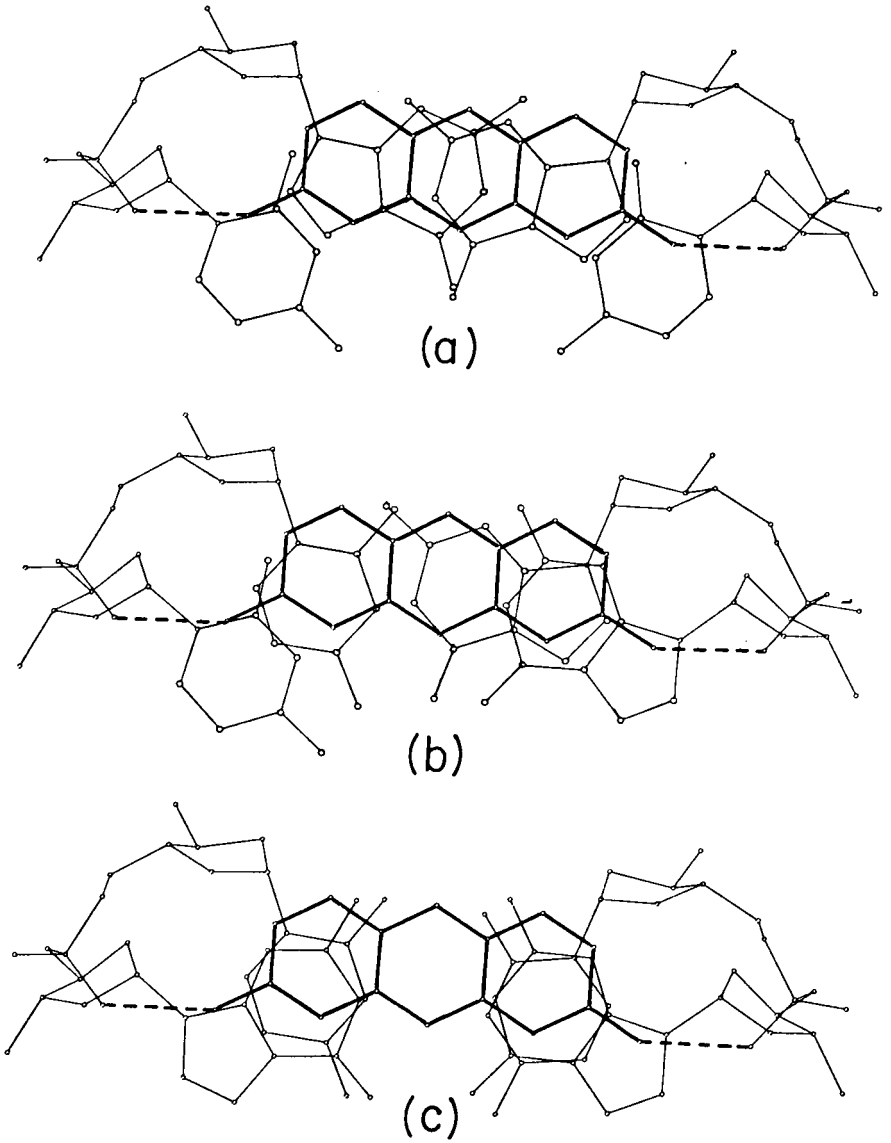


Figure 3.11. Stacking interactions between proflavine and A-DNA, with various base sequences [183]. (a) has (CpG)·(CpG), (b) has (CpG)·(CpG), and (c) has (GpC)·(GpC).

Computerised model-building has been used in attempts to define the stereochemistry of proflavine intercalation. As in the less precise models derived from fibre diffraction, these theoretical structures all have the helix axis constrained to be linear (as in the polynucleotides themselves). The model for B-DNA-proflavine intercalation (*Figure 3.9*) has the sugar conformation on the 5′-side of the intercalation site altered to be in a C3′-*endo* conformation [182]. Unwinding is over three residues, rather than the one of other models [31,34,181]; this helps to relieve steric strain. The total turn angle for these three residues is estimated to be 90°, equivalent to an unwinding of 18° per residue. It is considered that this concept of turn angle over the whole intercalated region is more meaningful than isolated unwinding angles. This is true both for closed circular DNA (which cannot have all residues with identical conformation on account of the topological constraints inherent in such a molecule), and for normal DNA, with alternating sequences (which preferentially bind intercalating drugs [48,105—107]) with non-standard conformations [11]. The same calculations have been performed on the A-DNA-proflavine intercalation complex [183], with similar results (*Figure 3.10*). The turn angle over the three residues around the intercalation site is 96.6°, corresponding to an unwinding of only 1.6° per residue for A-DNA. Unlike the B-DNA model, all sugars retain their (C3′-*endo*) original pucker. *Figure 3.11* shows that hydrogen bonds between proflavine amino groups and charged phosphate oxygen atoms, are most plausible. Base sequence (a) has the most base—drug overlap, in agreement with the observed sequence preference on binding [48]. It is pointed out that the A-DNA-proflavine conformation may well be adopted in a RNA complex — the two polynucleotides, as stated in an earlier section, are very similar. Furthermore, the A→B DNA polymorphism, which could be important in replication processes [184], may well be affected by the differing intercalative geometries.

It is noteworthy that none of these intercalation models (nor any discussed in the following section), provide evidence supporting a modification to the Lerman concept, in which the stacking interaction occurs between an acridine ring and two adjacent bases on the same polynucleotide chain [185], rather than with base pairs. However this model of partial intercalation should not be completely discounted; it may well be relevant to drugs such as daunomycin (3).

SINGLE-CRYSTAL ANALYSES OF MODEL COMPLEXES

Unlike fibres, crystals have three-dimensional ordering. This and other properties, enables X-ray diffraction patterns from them to yield, as least in

principle, detailed and definitive information concerning the three-dimensional structure of a molecular system. The techniques involved are beyond the scope of this review (see, for example, [186] for a non-mathematical introduction to the subject). The myth that solid-state conformations are not applicable in solution has been demolished for many macromolecules with the recent advent of high-field NMR methodology; for example, yeast phenylalanine transfer RNA has been clearly shown to maintain the tertiary structure found in the crystal, when in solution [187]. This is doubtless at least partly attributable to the co-crystallising of many biomolecules with appreciable quantities of water; their molecular environments are very aqueous and probably approximate to their solution environments which have semi-ordered water molecules clustered around. However, it must not be forgotten that diffraction methods in general provide static data. However much is learnt in the future about the structure of intercalation complexes, information about the dynamics of intercalative processes is gleaned only indirectly.

It has long been perceived that the simplest system capable of illustrating drug intercalation would be that of the structure of a (base-paired) dinucleoside, together with a drug. The actinomycin-deoxyguanosine crystal structure [120—123] only shows some of the features of interest; most others had to be conceived by extrapolation and model-building. The structure of a 9-aminoacridine-ApU (ApU = adenylyl (3′,5′)uridine) complex [188] has base-pairing, though not of the Watson-Crick type. This results in the dinucleoside not adopting a double helical-like conformation, and the overall structure not in fact being a model for intercalation. It is significant that the sequence of this dinucleoside is purine 3′,5′ pyrimidine, whereas 9-aminoacridine almost certainly binds preferentially to the reverse sequence [105,106]. Similarly, the proflavine-ApA(ApA = adenylyl(3′,5′)adenosine) complex does not form a double-helix structure [189]. Thus, the 'wrong' sequence in these model systems destabilises a double-helical structure, to favour any of a number of alternatives.

The crystal structure of a 2:2 complex between ethidium bromide and 5-iodo UpA [190—192] was the first to be determined that demonstrated true intercalative binding. The two dinucleosides are base-paired together to form a miniature double-helical-like fragment (*Figure 3.12*). One drug molecule is intercalated in-between and the other is stacked outside one of the base pairs; the crystallographic symmetry produces an ethidium molecule externally stacked on the other base pair. The complex as a whole has pseudo two-fold symmetry. The ribose sugars on each strand have alternating sugar pucker; both uridine residues have C3′-*endo* pucker whereas the adenosine ones have

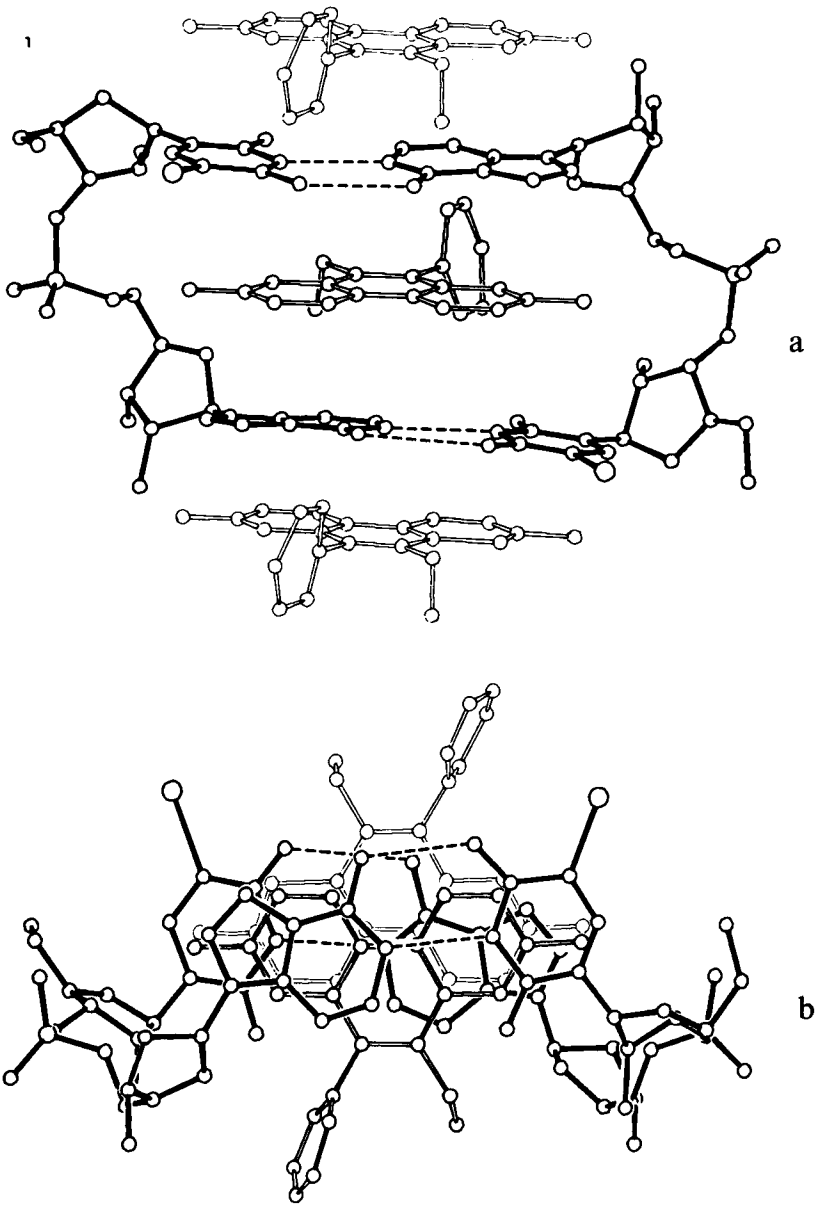


Figure 3.12. The ethidium-iodo UpA structure (a) viewed approximately parallel to the planes of the base pairs, and (b) viewed perpendicular to them. Dashed lines indicate hydrogen bonds [190].

C2′-*endo*. There are several other significant differences between this structure and the ethidium-DNA model derived from fibre diffraction [34]. For example, the 'unwinding' angle in the single-crystal study was judged to be 26° for RNA and 29° for DNA — the actual interglycosidic angle is 7°, compared with ones of 33° and 36° for RNA and DNA respectively. This contrasts with the estimate of 12° [34], but is in agreement with solution titration measurements on superhelical DNA [35]. The earlier model [34] has the drug intercalated from the wide groove of DNA, whereas in the single-crystal study the phenyl and ethyl substituents are pointing towards the narrow groove.

Crystal structures of several other drug-iodinated dinucleoside complexes have been reported. Ethidium-5-iodo-CpG(CpG = cytidylyl(3′,5′)guanosine) [193] closely resembles the UpA complex, whereas the 9-aminoacridine-5-iodo CpG one shows some interesting differences [194]. This structure has two distinct and unrelated base-paired fragments, each with 9-aminoacridine intercalated in-between. One miniature double helix has pseudo-symmetric intercalation whilst the other clearly does not; however, when external stacking is taken into account as well, similarities in overall acridine stacking properties have been noted. These, it is suggested, may be important in frameshift mutagenesis for stabilising looped-out regions of DNA. Analyses have also been made of 2:2 5-iodo-CpG complexes with ellipticine, acridine orange and proflavine [195]. In the case of the first two, the pattern of mixed sugar pucker and larger 'unwinding' angle is observed. However there is some confusion surrounding the conformation of the proflavine complex, which was initially reported [196] to have mixed sugar pucker and a 18° unwinding angle; subsequently [194] this was amended to all C3′-*endo* (or possibly C3′-*exo*—C3′-*endo* on just one strand [197]) with a 36° inter-glycosidic angle.

All these dinucleoside complexes, it will have been noted, have a covalently-bound iodine atom whose purpose is to facilitate the solution of the crystal structure, by the 'heavy-atom' method. However, the possibility that the iodine atom might perturb the conformation, particularly in the sugar region, cannot be discounted. Moreover, for all these analyses, accuracy of molecular geometry was relatively low, so interpretations of fine detail have to be made with care. Nevertheless, evidence in support of mixed sugar pucker for these intercalated dinucleosides has come from an NMR study [198] on propidium (10) complexed with both CpG and its deoxy analogue.

However, the analysis of a second proflavine-dinucleoside complex [199] has brought into question some of the conclusions and generalisations derived from these other structures [200]. Unlike these, the proflavine-CpG structure has no associated heavy-atom, and the accuracy of the analysis was appreciably higher. More importantly, the complex is a 3:2 one with exact

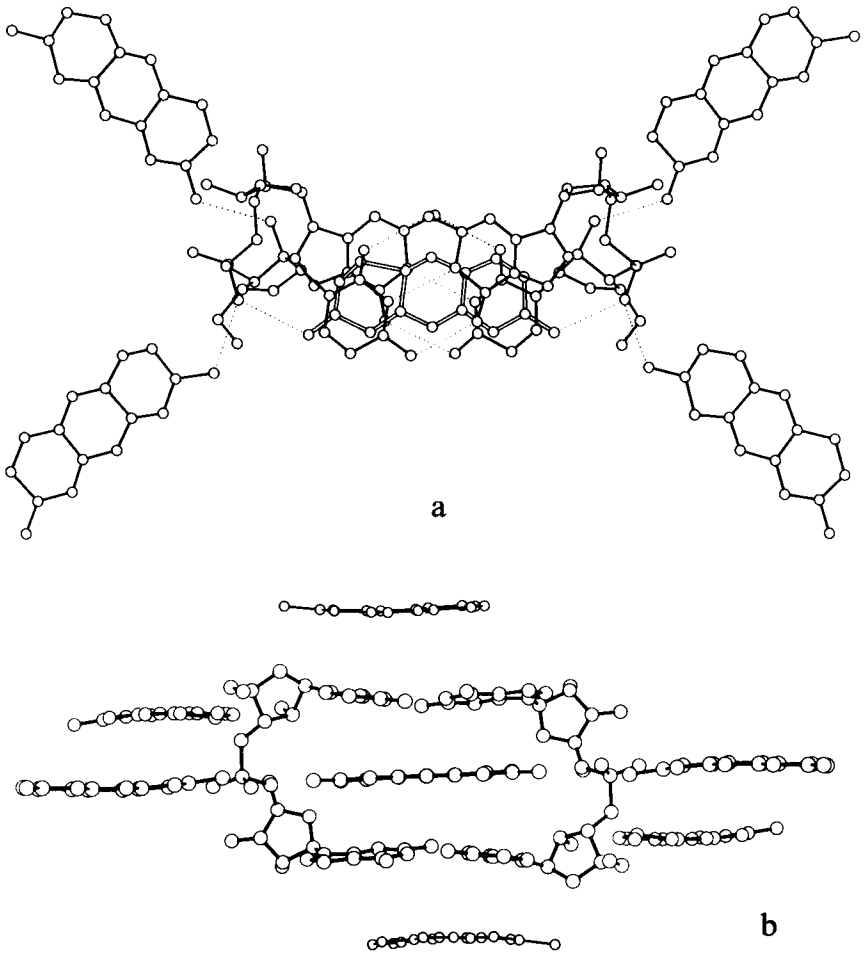


Figure 3.13. Two views of the proflavine-CpG complex [199], in (a) looking along the plane of the intercalated proflavine molecule with the bonds of the intercalated proflavine molecule shown unfilled and in (b) looking perpendicular to this plane. Dashed lines indicate hydrogen bonds.

two-fold symmetry, the symmetry axis passing through the central three atoms of the intercalated proflavine molecule (it is perpendicular to *Figure 3.13a*). The intercalated drug forms hydrogen bonds between its terminal nitrogen atoms and charged phosphate-oxygen atoms of the dinucleoside backbone. The other two proflavine molecules are hydrogen-bonded exter-

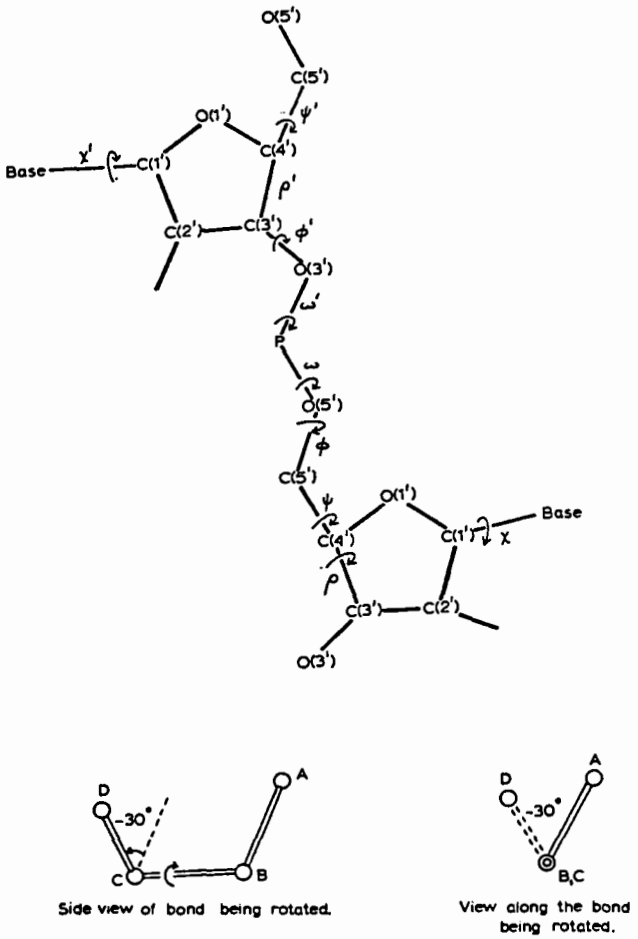


Figure 3.14. Conformational nomenclature for the nucleoside backbone (Courtesy of H.M. Berman).

nally, one to the same phosphate oxygen atom, the other to the cytidine ribose 02′ hydroxyl atom. Thus, this structure provides for the first time a simultaneous visualisation of the strong (intercalation) and weak (external) binding processes [21,23,27,41], albeit in a RNA and not a DNA analogue. Differences often observed between acridine binding to these two polynucleotides may well be explicable on the basis of external binding differences,

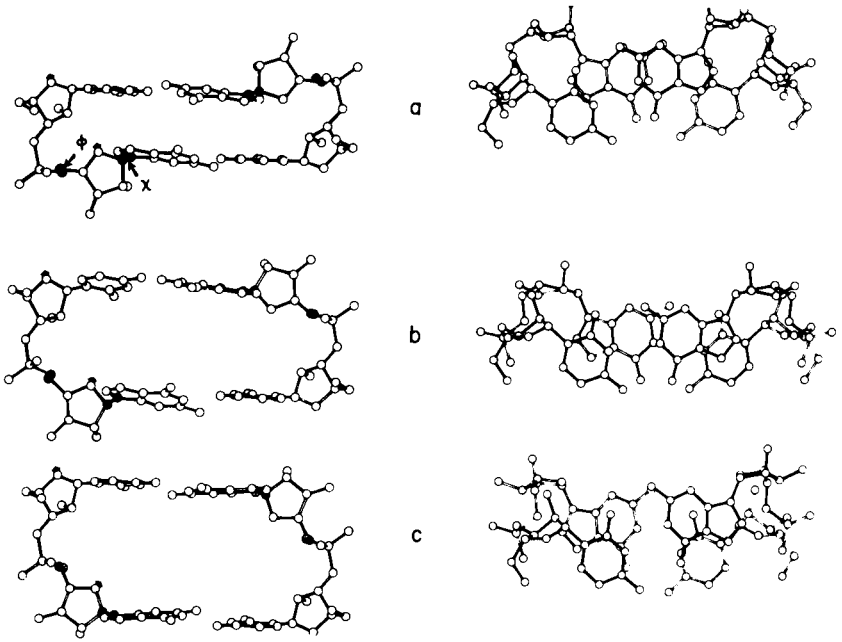


Figure 3.15. The stepwise stretching of a dinucleoside (CpG). (a) In a RNA-like conformation, (b) now with $\phi = 200°$, $\chi = 45°$, (c) a possible intercalation geometry with $\phi = 225°$, $\chi = 80°$. (Left) views parallel to the base planes; (right) views approximately perpendicular to the base-pair planes [201].

with only RNA being able to invoke the extensive external stacking and hydrogen-bonding of chromophores such as is seen in the model structure.

The complete retention of RNA C3′-*endo* sugar pucker in the proflavine CpG structure [199], together with an interglycosidic (sugar-base) angle of 33° (exactly that of RNA itself), has led to a study of the stereochemical basis of drug intercalation in these model systems [201]. This result [199] suggest that intercalation need not 'unwind' the miniature helix, a conclusion seemingly at variance with the accepted dogma of the subject. The conformation of the basic repeating unit is defined by a number of torsion angles (*Figure 3.14*). Examination of the crystal structures of all the intercalated dinucleosides has shown [201] that, regardless of the 'unwinding' angle or sugar pucker, only two of these backbone torsion angles alter significantly from their RNA eleven-fold double-helical values — these are ϕ and χ, which increase from 175° by about 60°, and from 13° by about 85°, respectively. Changes in the

other angles do occur, but they are small and non-systematic. Stages in this process are shown in *Figure 3.15*, as modelled on a computer. The effects of systematic changes in sugar pucker and interglycosidic vector angle (more appropriately termed in this context base-turn, rather than unwinding angle), were investigated by computer graphics simulations. Surprisingly, it was found that both these variables had but little effect on the dinucleoside backbone conformational angles (*Figure 3.14*). Instead, differences in geometry between, say, models with base-turn angles of 11° or 27°, or with either C3′-*endo*-(3′,5′)-C2′-*endo* or all C3′-*endo*, are reflected in small alterations in the base-pairing geometry. In particular, base pairs are easily able to become considerably twisted. Thus, it was concluded that sugar conformation in dinucleoside intercalated complexes can be of either identical or mixed pucker. A rough correlation between base-turn angle and steric bulk of the intercalating drug was observed [200], which could well be applicable to polynucleotides as well. Thus, ethidium bromide has bulky phenyl and ethyl substituents which force a large base-turn angle on intercalation, otherwise unacceptable van der Waal's contacts become produced.

Derived models of polynucleotide intercalation complexes

The models for proflavine intercalation into A- and B-DNA [182,183] obtained by computerised model-building, have a fundamental constraint of retention of the original polymer helix axis. In these, intercalation, *i.e.* opening-out of the base pairs at the binding site from 3.4 Å to 6.8 Å, is accomplished by alterations of the ψ and ω torsion angles (*Figure 3.14*). In contrast, intercalation into dinucleosides [201] involves changes in only the angles ϕ and χ. This alteration in χ (the glycosidic angle) produces a disruption in any helix produced from these models making them non-linear.

This disruption of helicity has been identified [200] as simultaneous kinking of the base pairs at the intercalation site by about 8° (for ethidium), and dislocation of the helix axis. These features have been extended to a drug-DNA model; it is considered that a similar helical screw axis dislocation is produced in DNA, made possible by altering the normal C2′-*endo* sugar pucker to a mixed puckering pattern, as described above. This C3′-*endo*-(3′,5′)-C2′-*endo* pattern necessarily predicts binding at every other site, *i.e.* neighbour exclusion. The DNA-ethidium model possesses a slow right-handed superhelical structure. The concept of kinking developed in this study has been utilised in an attempt to develop a model for the organisation of DNA in chromatin [202]. This has kinking every ten base pairs, which results in a left-handed superhelix.

This intercalation model has been questioned by a further analysis of the dinucleoside models [203]. It has been found that, contrary to the previous experience [200], building of a polymer is not straightforward. This difficulty primarily arises from the marked non-equivalence of the two glycosidic angles χ and χ' in the intercalated dinucleosides—a continuous, linear polynucleotide (DNA or RNA) double helix necessarily has these angles equal, with the polymer repeating unit being a mononucleotide. The concept of kinking outlined above [200,202] implicitly acknowledges this polymer discontinuity. In view of all the dinucleosides in these model structures having ribose rather than deoxyribose sugars, they must be representative of the RNA situation rather than the DNA one. (It may be noted that the uncomplexed dinucleosides GpC and ApU form helical fragments in the solid-state with double-helical RNA characteristics [204—206]). Thus, a model of proflavine intercalation into RNA has been constructed [203] with these constraints and having an alternating C·G sequence on each strand. Since all the intercalated dinucleoside structures have an invarient backbone conformation [201], a CpG duplex with this structure was taken as a starting point and the alternating sequence was added on both ends to result in the tetranucleotide GpCpGpC. Computerised model-building showed the difficulty of base-pairing the ends while maintaining the central intercalated geometry (in marked contrast to the apparent ease of polymer model-building previously encountered [200,202]). The final structure has a marked asymmetry in the base pairs adjacent to the intercalation site, which necessarily excludes nearest-neighbour intercalation. Thus this model predicts an extended excluded site resultant of intercalation into RNA; this agrees with data obtained for diacridines intercalated into RNA [178]. Whereas several diacridines could easily bis-intercalate into DNA, binding to RNA was strictly monofunctional. Similarly, the antibiotic echinomycin, also a bifunctional intercalator into DNA but one without flexibility of interchromophore linkage, does not significantly bind to RNA or synthetic polynucleotides [164,165]. It is notable that the asymmetry of this model [203] involves two adjacent phosphate groups near the binding site becoming rather closer together than in the uncomplexed polymer—the resulting 'hump' in the backbone could well function as a recognition site for nuclease attack and subsequent *in vivo* strand breakage. It has also been possible to construct [203] a neighbour-exclusion site model which has the base pairs adjacent to the intercalation site relatively steeply inclined to those at the site itself.

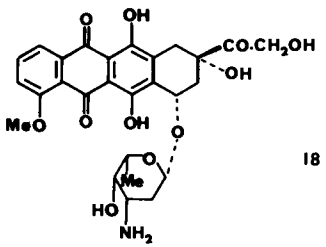
Since at present, no structural information is available on deoxyoligonucleotide intercalation complexes, it seems premature to speculate on detailed stereochemistry of the DNA complexes. DNA in principle has much

greater conformational flexibility than RNA — the loss of the 2′-hydroxyl group on the pentose sugar in the former removes many steric restrictions to conformational mobility. Thus, there are *a priore* no grounds for assuming that the standard dinucleoside intercalation geometry [201] would be maintained even in a dideoxynucleoside. The adoption of an unexpected, non-classical DNA conformation by the deoxytetranucleotide d(pApTpApT) [207] underlines this point. In this structure, the sugar pucker and glycosidic angle are remarkably 'soft' parameters.

The situation *in vivo* is rather more complex since DNA in chromosomes exists in a folded form with associated nucleoproteins [208], rather than as straight double helices. Various models have been suggested for the structure of the DNA in the nucleosome subunit [200,209,210]; it is clear that the classical B-DNA structure [6—8] is an inadequate representation of the conformation, although one will have to await appropriate experimental data becoming available before precise details are known. The implications for an understanding of drug intercalation into DNA are apparent and suggest that a cautious approach is appropriate at present when visualising these processess.

DAUNOMYCIN AND RELATED COMPOUNDS [210a]

The antibiotic daunomycin (daunorubicin) (3) and its 14-hydroxy derivative adriamycin (doxorubicin) (18) are highly cytotoxic compounds isolated from



18

strains of *Streptomyces peucetius* [211—213]. Whereas the parent compound is restricted in its clinical usefulness to treatment of acute lymphocytic and myelogenous leukemia, adriamycin is remarkable for its exceptionally wide spectrum of activity. It displays significant effects against a variety of solid tumours, including several previously unresponsive to chemotherapy [214].

These two drugs and most of its derivatives are believed to exert their primary effect of inhibiting neoplastic cell growth by interacting directly with DNA [211—213,215]. However it must be emphasised that a variety of other effects have been noted. Indeed, in view of the complex multifunctional chemistry of these antibiotics, it would be surprising if the *in vivo* mechanisms of action were other than complex. Nevertheless, the most plausible hypothesis of the primary action of the anthracycline anti-cancer agents, remains a DNA-binding one [216,217]. In view of the widespread acceptance of this view and of the actual and potential importance of the drugs and their derivatives, a large body of data has been accumulated on structure-activity and DNA-binding properties [212,215,218,219], much of which has shed further light on intercalation processes generally.

BIOLOGICAL ACTION

The *in vitro* growth of a number of normal and neoplastic cell lines is markedly inhibited by daunomycin and adriamycin [211,213]. This inhibition is characterised ultrastructurally by cell damage, chiefly to the nucleus [220]. Phase contrast and electron microscopy, and autoradiography have shown that chromosomal binding and damage is the primary effect produced [221]. Subcellular localisation of adriamycin and daunomycin in cultured fibroblasts [222] has shown exclusive uptake in nuclei and lysosomes, with a higher proportion of the former drug being localised in the nucleus (in agreement with its greater specific inhibition of cell growth). Similar differences between the two drugs have been found for Ehrlich ascites tumour cells [223] — in this case about 70% of the total daunomycin binds to the cell nucleus.

Both RNA [224—229] and DNA synthesis [229—231] are inhibited by these drugs in cell cultures and in intact animals as well as in cell-free systems. Studies on isolated polymerase systems have shown that the inhibitions are not due to drug-polymerase interactions. With *E. Coli* DNA-dependent RNA polymerase [224], the inhibition is independent of polymerase concentration and is consistent with drug-template (*i.e.* DNA) binding. This conclusion is in accord with results on intact L1210 mouse leukemia cells [225], which also showed that DNA and RNA metabolism were inhibited to the same extent (although some differences between the two have been observed [229]). Protein synthesis was unaffected by the anthracyclines. Further evidence for their direct template binding has come from studies on inhibition of DNAase I activity [232]; only those derivatives of daunomycin which formed complexes with DNA inhibited enzymatic activity.

In general, the extent of inhibition of nucleic acid synthesis (in a suitable test

Table 3.3. BIOLOGICAL ACITIVITY OF SOME DAUNOMYCIN DERIVATIVES [215]

Compound	*Dose required for 50% inhibition of DNA synthesis (mol × 10^6)*	*Average survival time[c] for S180 ascites experimental tumour*
Daunomycin (3)	1.6[a]	222[a]
Adriamycin (18)	3.4[a]	227[a]
13-Dihydrodaunomycin	8.8[a]	231[a]
Daunomycin-13-semicarbazone	> 8.5[a]	
Daunomycin-13-oxime	> 8.6[a]	130[a]
N-Acetyldaunomycin	> 8.3[a]	100[a,d]
N-Guanidineacetyldaunomycin	> 7.6[a]	100[a]
Adriamycin 14-octanoate		250[b]
Adriamycin 14-acetate		252[b]
2-Amino-2-deoxyglucosyldaunomycinone (20)	8.8	107[a]
4′-Epidaunomycin	9.0[e]	234[f]
β-Anomer of adriamycin	> 40[e]	
β-Anomer of 4′-epidaunomycin (21)	> 40[e]	126[f]
4-Demethoxydaunomycin		264[g]
N-Trifluoroacetyladriamycin 14-valerate (AD32)	> 50[h]	> 400[i]
Daunomycin 13-benzhydrazone (rubidazone)	2.0[i]	

[a]From [211—213]. [b]From [212]. [c]Average survival times as percentage of controls. [d]From [233] results are for the inhibition of growth of Rous Sarcoma Virus. [e]From [234]. Note that this study reports values of 6 and 8 μM for adriamycin and daunomycin 50% inhibition of RNA polymerase (contrast [211]). Values given here are thus not strictly comparable with others in the table[f]. From [235] optimum dosage average survival times are given. [g]From [236]. [h]From [237]. [i]From [238]. This represents a median percentage increase in survival time for both P388 and L1210 leukemia. Derivative numbering is given in formula (19).

system) can often be correlated with biological activity against experimental tumours. *Table 3.3* details such data accumulated for a number of daunomycin derivatives. Due to differences between test systems and conditions, not all the data are quantitatively comparable. However, qualitative trends are apparent.

BINDING TO DNA

The *in vitro* binding of daunomycin and its cogeners to DNA has been the subject of numerous investigations [215]. Lack of standardisation in experimental conditions and methodology has produced a surprisingly wide

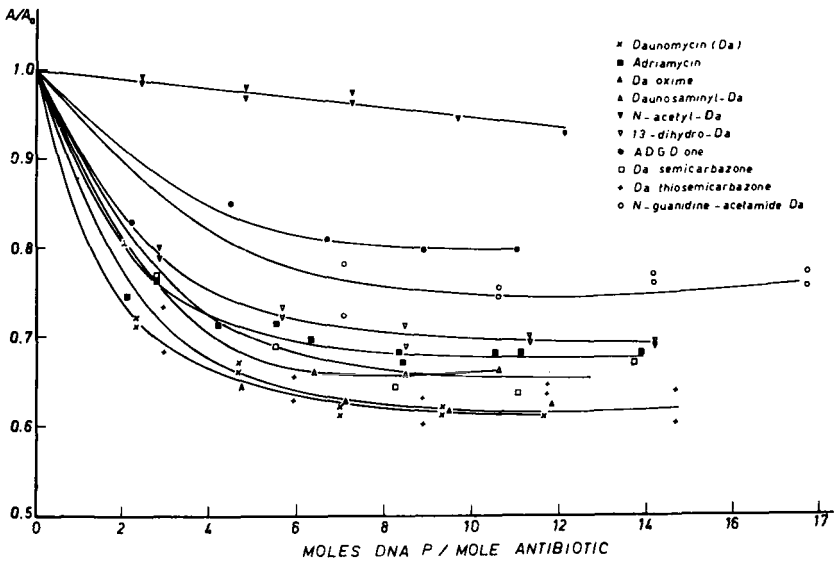
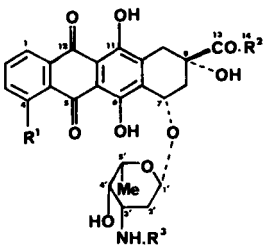


Figure 3.16. The effect of calf thymus DNA on the 480 nm absorption maximum of daunomycin and various derivatives. The antibiotics were at 0.5 × 10^{-4} molar concentration, buffered at pH7. ADGDone is derivative (20) [241].

spectrum of numerical binding data, ensuring that reliable comparisons are difficult to make.

Spectroscopic methods have been extensively employed. The 480 nm visible absorption maximum of daunomycin is decreased on addition of DNA and is shifted to longer wavelengths (505 nm at a DNA: drug ratio of 7:1) [239,240]. The ultra-violet maxima similarly shift, but to shorter wavelengths. As *Figure 3.16* shows, a wide variation of behaviour has been observed, with daunomycin itself showing a maximal effect and derivatives such as the *N*-acetate (19, $R^1 = 0Me$, $R^2 = Me$, $R^3 = Ac$) a minimal one.

19



The characteristic fluorescence of the anthracycline chromophore in these compounds is quenched when binding to DNA takes place [234, 239, 242, 243]. This effect has been monitored (rather than visible absorbance changes) when investigating derivatives for which only small quantities are available. This situation is not uncommon since daunomycin and adriamycin are exceptionally expensive drugs. Other spectral techniques such as circular dichroism have been but little used; this is possibly because of the difficulties involved in interpretation of data. Interaction of daunomycin with DNA produces characteristic alterations in the drug's CD spectrum [244—246], which vary with different derivatives (though in a complex manner).

A progressive increase in the relative viscosity of DNA is produced upon binding of daunomycin [239,240,247], which is analogous to that produced by proflavine and other intercalating agents. Viscosity changes have been monitored for a number of daunomycin derivatives [241], with effects that are broadly comparable with those in *Figure 3.16*. The sedimentation coefficient and buoyant density of DNA are decreased on binding of daunomycin; in contrast to the simpler DNA-binding agents, the complex formed has been reported to be relatively stable at high ionic strength. The melting temperature of DNA (T_m) is substantially increased from 70.5°C to 83.9°C [240] on binding the drug, at a 1 : 10 drug : nucleotide ratio. This indication of enhanced helical stabilisation on binding has frequently been employed as a measure of the relative properties of daunomycin derivatives [212,218]. Equilibrium dialysis techniques have been used to obtain binding parameters [240,245,248], and from such measurements it has been estimated that the binding, as measured by the association constant K_{app}, is not markedly dependent on ionic strength [248].

Daunomycin removes and reverses the supercoiling of closed-circular DNA [33], although the unwinding angle found is less than half that of ethidium—5.2° relative to 12° for ethidium. Adriamycin has a similar unwinding angle of 5.3° [249]. These low values have been taken to indicate that only about 44% of the drug is bound, in conflict with another, perhaps less reliable study which finds almost all of the drug bound, yet with only a 4° unwinding angle.

Quantitative analysis has been made of the bindings of a number of derivatives of daunomycin (*Tables 3.4 and 3.5*). As mentioned above, considerable discrepancies between different studies have emerged [215]. Thus the association constant for the daunomycin-DNA interaction has been reported to be as low as 1.3×10^6 mol^{-1} [250] and as high as 9.3×10^6 mol^{-1} [245]. It is likely that the lower values recorded [215] are more correct, a conclusion reinforced by a recent thorough analysis [251] using computerised

curve-fitting to both direct and difference visible spectra. This study finds significant differences between daunomycin and adriamycin binding, in contrast to some earlier measurements [240,243]. In all cases, the apparent number of binding sites corresponds to one drug molecule per six nucleotides (*i.e.* every three base pairs).

The Scatchard plots obtained from the binding analyses are typically non-linear, indicating more than one mode of binding [240]. This has also been shown by a study of the elution behaviour of daunomycin on DNA—cellulose columns [257]. Taken together, all these results correspond to the behaviour expected for an intercalating drug. Thus, the strong initial binding observed is considered to be due to the anthracycline chromophore intercalating into DNA; associated with this stabilisation is an electrostatic interaction

Table 3.4. APPARENT BINDING CONSTANTS (K_{app}) AND NUMBER OF BINDING SITES PER NUCLEOTIDE (B_{app}) FOR VARIOUS DAUNOMYCIN DERIVATIVES

Compound	K_{app} ($mol^{-1} \times 10^{-6}$)	B_{app}	*Reference*
Daunomycin (3)	3.8	0.17	(a)
	1.3		[251]
Adriamycin (18)	3.0	0.19	(a)
	2.0		[251]
13-Dihydrodaunomycin	1.1	0.10	[240]
N-Acetyldaunomycin	1.8×10^{-2}	0.12	[240]
N-Guanidineacetyldaunomycin	72	0.09	[240]
2-Amino-2-deoxyglucosyl daunomycin (20)	7.1×10^{-2}	0.09	[240]
4′-Epiadriamycin	2.2	0.24	[234]
4′-Epidaunomycin	2.0	0.19	[234]
4-Demethoxydaunomycin	2.4	0.20	[242]
Daunomycin 13-benzhydrazone	7.0[b]	0.18	[245]
Daunomycin D-*N*-Me_2glycine	1.7[b]	0.18	[245]
3′-Epidaunomycin	0.9	0.22	[252]
3′,4′-Epidaunomycin	0.9	0.20	[252]
4′-Deoxydaunomycin	2.5		[253]
2′-Hydroxydaunomycin	1.3[c]		[253]
6′-Hydroxydaunomycin	1.2[c,d]		[253]
14-Morpholinodaunomycin	2.1[e]		[219]

[a]Averages from [215].
[b]Relative to a K_{app} for daunomycin of $6.8 \times 10^6 mol^{-1}$.
[c]Relative to a K_{app} for daunomycin of $2.3 \times 10^6 mol^{-1}$.
[d]The 6′-hydroxyl group is attached to the 5′-methyl.
[e]Relative to a K_{app} for daunomycin of $4.5 \times 10^6 mol^{-1}$.

Table 3.5. THE CHANGE IN THERMAL TRANSITION TEMPERATURE (ΔT_m) IN °C, FOR VARIOUS DAUNOMYCIN DERIVATIVES[a]

Compound	ΔT_m	*Reference*
Daunomycin (3)	13.4	[240]
Adriamycin (18)	14.8	[240]
N-Guanidineacetyldaunomycin	8.3	[240]
N-Acetyldaunomycin	1.0	[240]
2-Amino-2-deoxyglucosyl-daunomycinone [20]	8.0	[240]
4′-Epidaunomycin	12.4	[234]
4′-Epiadriamycin	12.5	[234]
β-Adriamycin	3.0[b]	[234]
β-Anomer of 4′-epidaunomycin (21)	4.8	[234]
β-Anomer of 4′-epiadriamycin	7.6	[234]
4-Demethoxydaunomycin	21	[242]
4-Demethoxy-7,9-bis-epidaunomycin (22)	10	[242]
β-Anomer of 4-demethoxydaunomycin	18	[242]
β-Anomer of 4-demethoxy-7,9-bis-epidaunomycin (23)	7	[242]
Compound (24)	0.5[c]	[218]
Compound (25)	2.2[c]	[218]
Compound (26)	6.3[c]	[218]
13-Deoxydaunomycin	9.8[d] (5.0)	[240] ([255])
N-Trifluoroacetyladriamycin 14-valerate	0[e]	[256]

[a]Relative to a T_m of 70.5° for DNA alone.
[b]4.5°C from [254].
[c]Relative to ΔT_m for adriamycin of 17.8°C [218].
[d]Relative to ΔT_m for daunomycin of 11.2°C [255].
[e]Relative to ΔT_m for adriamycin of 11°C [256].

between the protonated amino group of the sugar residue and the negatively charged phosphate oxygen atoms of the polynucleotide backbone. Thus, *N*-acylation markedly decreases the drug's binding to DNA and does not stabilise the double helix against thermal denaturation. In general, *N*-acylation has a deleterious effect on *in vivo* nucleic acid synthesis and antitumour action (though there are exceptions to this [238]). The dependence of binding on ionic strength would be expected to be substantial, as has indeed been found [239]; however, equilibrium dialysis measurements have contradicted this [248].

The base and sequence dependence of daunomycin-DNA binding has been the subject of several studies. However, as yet, no clear picture has emerged from them. Thus, the slight dependence of buoyant density on G·C base composition [247] is not paralleled by the degree of inhibition of DNA-

dependent RNA polymerase on various templates [227] or by analysis of melting curves for DNAs with varying base composition [254]. Other melting curves [224,257] show that stabilisation of double-helical structure (measured by ΔT_m values) increases with increasing A + T content. Interestingly, considerable differences were observed in ΔT_m between behaviour with the alternating polydeoxynucleotides (*e.g.* poly(dA·dT)·poly(dA·dT)) compared with the non-alternating ones (*e.g.* poly(dA·dT)) [257], with the former being much more stabilised. These differences, which were not revealed by the K_{app} values, could well be related to the differences in conformation between the two types of polymer [8]. These results are in conflict with those of a previous study [243]. Actinomycin, which by itself does not bind to poly(dA·dT)·poly(dA·dT), does so in the presence of daunomycin [246].

Daunomycin does not bind to double-stranded RNA or synthetic polynucleotides in an intercalative manner [250,258]; the B-DNA type structure is thus a requirement for daunomycin—polynucleotide intercalation, in common with the other relatively complex drugs, actinomycin and echinomycin. On the other hand, daunomycin binds very strongly to (unfractionated) transfer RNA [259], with a K_{app} of 1.2×10^8 mol^{-1} and about three binding sites per tRNA molecule. One suspects that the binding is non-intercalative, with the drug being constrained in folds of the tertiary structure, as has been found for ethidium [104]. In support of the hypothesis that tRNA is indeed a cellular receptor for daunomycin, it has been reported that the drug does inhibit *in vitro* protein synthesis [260], although other studies do not confirm this [225,229].

MOLECULAR MODELS OF DNA BINDING

The conformation of the drug

The crystallographic analysis of the *N*-bromoacetyl derivative of daunomycin [261] verified the molecular structure and relative stereochemistry of the drug, previously established on the basis of extensive chemical studies [211]. This study was of low accuracy due to poor crystal quality and solvent disorder.

More recently, the crystal structure of carminomycin I (4-hydroxydaunomycin), as the hydrochloride monohydrate, has been subjected to two independent crystallographic analyses [262—264]. Crystals of daunomycin itself has also been analysed, both as a pyridine adduct of the hydrochloride monohydrate [265] and as a hydrochloride butanol adduct [266]. The drug conformations found in all except the last-named study have been compared and analysed [265]. *Figure 3.17* shows that there is an overall close

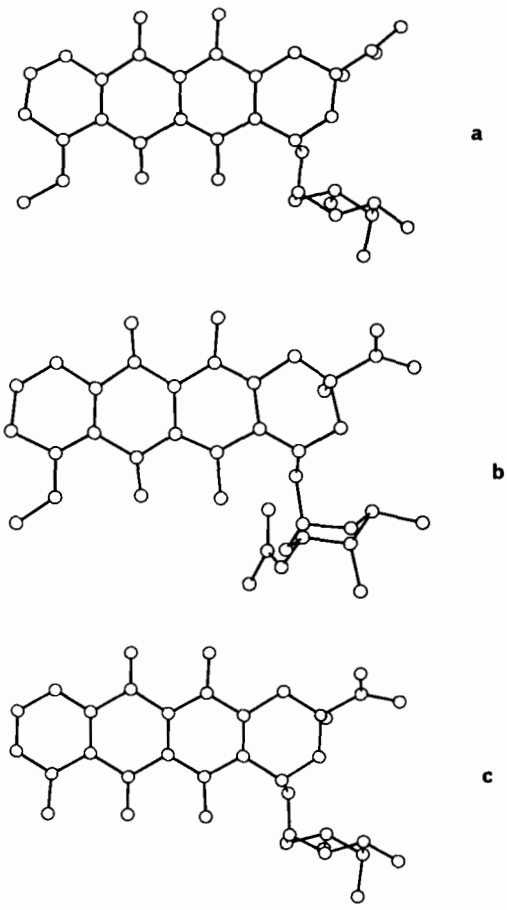


Figure 3.17. Computer-drawn projections, looking down onto the anthroquinone plane of (a) daunomycin, in the daunomycin-pyridine crystal structure [265], (b) N-bromoacetyl-daunomycin [261] and (c) carminomycin I, drawn from the averaged coordinates of [262—265].

correspondence between the stereochemistry of carminomycin and daunomycin (in its pyridine adduct), with some differences of detail being apparent between these and that of the *N*-bromoacetyl derivative. These differences are most manifest in the cyclohexene ring A puckering and in the relative dispositions of the daunosamine (sugar) ring. At a gross level, all the conformations found are remarkably similar, in spite of the apparent large degree of flexibility around the glycosidic linkages. It was originally suggested

[215,265] that this stabilisation was due to an intra-molecular hydrogen bond from O_9 to the glycosidic oxygen atom, which has actually been observed in a crystal structure [264]. However, semi-empirical potential-energy calculations have revealed a surprising lack of flexibility around the glycosidic linkages [267] — the conformation found in the solid state for daunomycin indeed corresponds to its energetically-favoured structure. The hydrogen bond merely serves to strengthen this preference.

These results render relatively unlikely an alternative conformation proposed [218] for daunomycin on the basis of a hypothetical DNA-binding model. In this alternative, the daunosamine ring and the C-13 side-chain are equatorial and axial respectively to ring A of the chromophore. These are the reverse of the crystallographic assignments of configuration.

The existence of a preferred conformation for a flexible molecule, as indicated by X-ray studies in the solid-state, must in general be treated with caution when extrapolated to the situation in solution. However, since daunomycin has been found to adopt the same conformation in three non-identical crystallographic environments (a structure supported by energy calculations), it has been suggested that this conformation is the biologically active one in solution (except for the flexible C-13 side chain) [265].

Models for daunomycin intercalation

Analysis of X-ray fibre diffraction patterns of a daunomycin-DNA complex [268] has provided a binding model that has received widespread acceptance [211—213]. However, as has been discussed in earlier sections, fibre diffraction analysis suffers from defects of inherent lack of fine detail in the final molecular model obtained. So, data concerning the stereochemistry of nucleotides in the intercalation region, as well as a definitive confirmation of the model itself, must await a single-crystal analysis of a suitable model system.

The best fit to the data was obtained with a 12° unwinding model, with the daunosamine ring situated in the wide groove of the double helix (*Figures 3.18 and 3.19*). The drug conformation was that found for the *N*-bromoacetyl derivative [261], which from the argument in the previous section, is a fair assumption. The sugar ring is in such a position that the charged nitrogen atom at C-3′ forms a close contact with an anionic phosphate oxygen atom, two residues away from the intercalation site. The model thus explains the lack of binding of *N*-acetyl derivatives and has the drug bound every three base pairs.

The subsequent modifications to the definition of daunomycin confor-

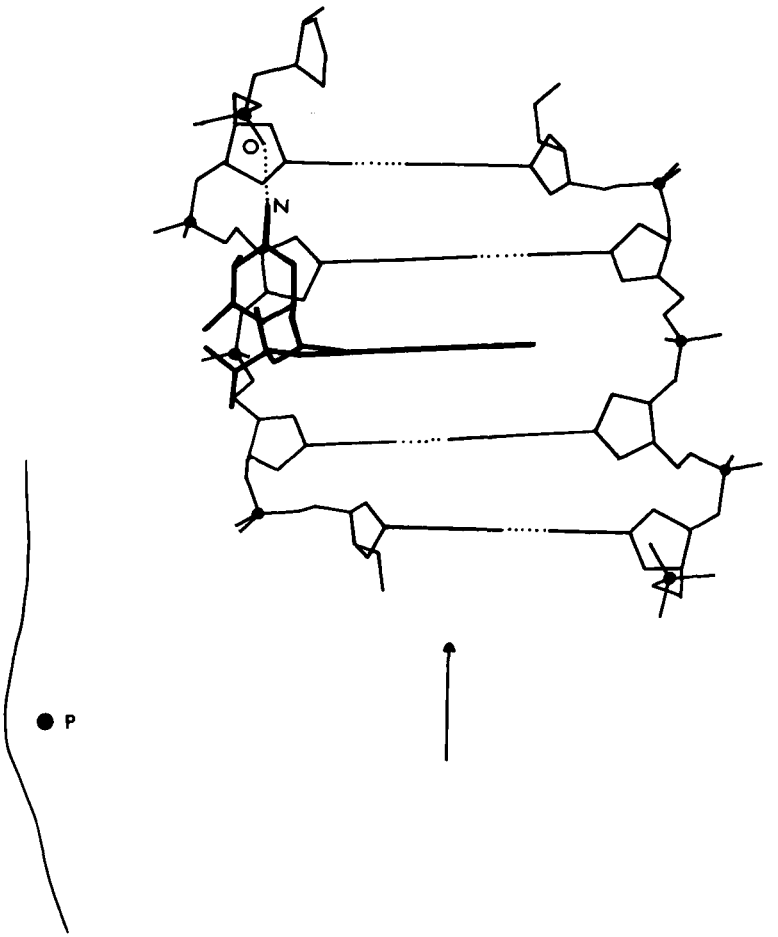


Figure 3.18. A representation of the modified DNA-daunomycin binding model, with the drug molecule shown in heavy outline. The daunomycin conformation is as described in [265]. The arrow indicates the helix axis [215].

mation [262—265] have made relatively little difference to this model. The probability of the ethidium unwinding angle being 26° rather than 12° [35] results in the daunomycin unwinding angle being 11° rather than 5.2° [33], close to the 12° value chosen in the model-building [268].

An alternative intercalation model has been proposed [218], based on the hypothetical daunomycin conformation discussed above. It utilises the

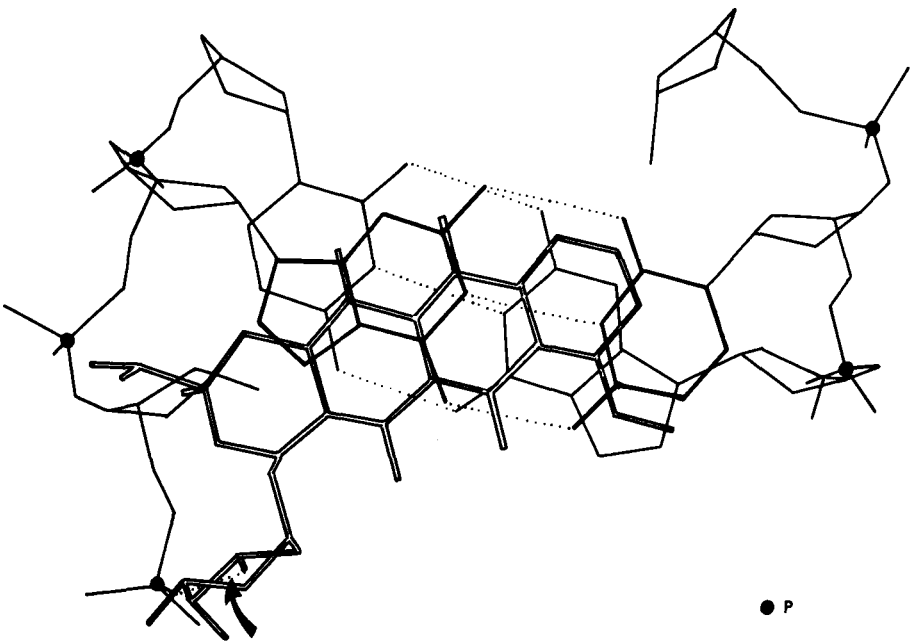


Figure 3.19. As in Figure 3.18 but now viewed looking down onto the planes of the base pairs. The upper base pair at the intercalation site is drawn in heavy outline, and the drug molecule in double outline. The arrow indicates the nitrogen-oxygen hydrogen bond [215].

maximum possible number of drug functional groups in hydrogen bonds both to the DNA backbone and to bases themselves (for which there does not seem to be experimental evidence). The model also implies that only two base pairs are involved in a binding site.

A detailed examination of the Pigram—Fuller—Hamilton proposal [268] has suggested that adriamycin (18) might be additionally stabilised relative to daunomycin (3) by an additional hydrogen bond from the C-14 hydroxyl group to the phosphate group at the intercalation site [269]. It may be significant that the recent accurate determination of binding constants for the two drugs [251] suggests that adriamycin binds DNA slightly more strongly; ΔT_m data (*Table 3.5*) also supports this. Furthermore, a higher proportion of adriamycin than daunomycin binds to the cell nucleus *in vivo* [222]. These models suffers from many of the deficiencies common to the other intercalation structures presented in this review. Nevertheless, they have been successful in explaining a large body of the binding data for daunomycin

derivatives. A more detailed model, together with unequivocal data on sequence preferences for binding, is undoubtedly required.

Relationships to properties of derivatives

It is apparent [218] that a maximum size of chromophore is needed for effective intercalative binding, as shown by studies on model analogues of daunomycin. Synthetic compounds with two or sometimes even three rings do not stabilise the DNA helix to any appreciable extent. Compound (24) is required in a concentration of 210 μM in order to effect a 50% reduction in RNA synthesis, compared with a concentration of 0.3 μM for daunomycin. This requirement for a minimum size of intercalative chromophore is entirely predictable from the binding model (see especially *Figure 3.19*), in terms of minimum base pair—chromophore overlap. A series of three-ring, amino-alkylaminoanthraquinone analogues has been examined [270,271]. Those with diaminoalkyl substituents bound strongly to DNA; in one case, the association constant was higher than for the parent drugs. No biological data have been reported for these analogues; it will undoubtedly be of interest to ascertain whether their strong DNA binding correlates with high activity.

The daunomycin-binding model predicts that partial or complete removal of the 4-demethoxy substituent on ring D (19) could result in slightly increased penetration of the chromophore into the intercalation site, since the steric constraints of the bulky methoxy group would be removed. 4-Demethoxydaunomycin stabilises the DNA helix to a greater extent that does the parent drug [236,242], even though its association constant for the binding is slightly lower. It has been suggested that the absence of the 4-methoxy group produces alterations in the electronic properties of the chromophore [272], which one would expect to see reflected in binding properties. The 4-demethoxy derivatives of both daunomycin and adriamycin have excited considerable current interest [273] since they are as effective as the parent drugs when tested against a wide variety of experimental tumour systems [219,236,274,275], but at dose levels up to ten times lower than either daunomycin or adriamycin, *i.e.*, they are markedly more potent. The derivatives (and their 4′-epi isomers) may well show differential tumour effects, of possible clinical use. The severe cardiotoxic side-effects of daunomycin and adriamycin (to which further reference will be made in the subsequent section) are dosage-dependent; so drugs such as the 4-demethoxy derivatives may well hold out useful clinical promise. Carminomycin I, the 4-hydroxy derivative, has also been reported to show enhanced potency [218].

Modifications of the C-9 side chain have been especially favoured in studies

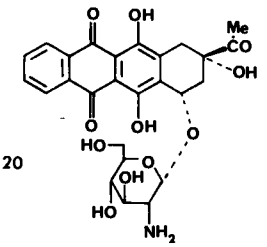
of daunomycin derivatives. Examination of the molecular model shows that this side-chain protrudes out from the intercalation site (*Figure 3.19*); thus, provided no too bulky groups are attached, substitution here would not have profound effects on binding, although they might somewhat deleterious — this is illustrated by the reduced activity and binding ability of C-13 derivatives such as the oxime or semicarbazone. Pharmacokinetic properties may well be enhanced by substitution here. The 14-*O*-acyl derivatives, such as the 14-octanoate do indeed show enhanced activities (*Table 3.3*).

It has been suggested [269] that substitution of a highly basic group at C-14, such as $-NH_2$ or even $-NH_3^+$, would enhance DNA-binding affinity, in line with the hypothesis of enhanced adriamycin stabilisation compared to daunomycin. The secondary amine derivatives such as the 14-morpholino and 14-piperidino [219] show reduced DNA binding, though comparable potency (albeit at higher dosage levels), compared with the parent daunomycin. However, this claim of significant activity levels has been refuted by other workers [276]. It is clear though, when the molecular models are examined, that all of these secondary amines cannot fulfil the required conditions for enhanced binding [269] and are anyway too bulky to fit into the small area allowed (*Figure 3.19*). A series of bis-adriamycin analogues has been synthesised [277]; these have two adriamycin molecules linked through C-13 by various amino acid-type chains. Some of these compounds show high anti-cancer potency and good efficacy. It is unclear at present whether they indeed bis-intercalate into DNA; bis-daunomycin and adriamycin analogues of this or any other type can be expected to be of considerable interest in the future in view of the current studies in bis-intercalation generally.

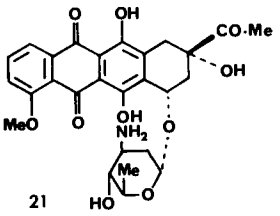
The effects of altering the absolute stereochemistry of daunomycin at C-7 and C-9 (19) have been examined [236,242]. The 7,9-bis-epi compounds (*cf.* (22)) have low DNA-binding affinities and zero or slight biological activity. These results can be understood in terms of the molecular model for binding since these derivatives, whatever their precise conformations, clearly cannot fit into the daunomycin binding site in DNA. Their 3′-amino group, in particular, cannot interact with a phosphate group as it does in daunomycin.

Modifications to the sugar ring of daunomycin have been extensively investigated. In particular, the highly stereospecific requirements of the 3′-amino group at the DNA binding site have been well documented. 2-Amino-2-deoxyglucosyl daunomycinone (20) has poor binding properties and biological activity, as has 3′-epi-daunomycin [252].

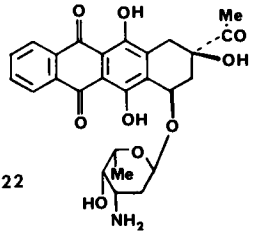
Inversion of configuration at C-1′ produces the β daunomycin derivatives [234,242,278] such as (21) and (23). These probably cannot bind to DNA in



20



21



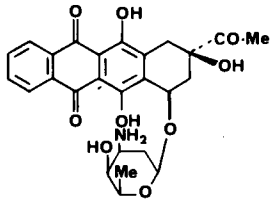
22

the same way as daunomycin, since the N-3′-phosphate close contact cannot be made in an analogous manner. Although accurate binding parameters have not been obtained for these derivatives, they certainly bind much more weakly to DNA than do the normal *a* ones and have reduced anti-tumour activity.

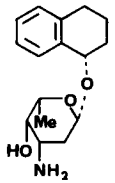
The 4′-epi derivatives, both of daunomycin and adriamycin and of their 4-demethoxy derivatives, show promising anti-tumour activity [219,234,235]. They are significantly less toxic to cultured cardiac cells, possibly indicating a different mode of pharmacodynamic transport across membrane boundaries [219]. The 4′-deoxy derivative [253] shows a slightly stronger binding ability to DNA than does daunomycin and it has higher biological activity. All these results suggests that the 4′-hydroxyl group is but little, if at all, involved in the binding to DNA, a conclusion supported by the Pigram—Fuller—Hamilton model [268].

Semi-synthetic analogues such as (26) [218], although they show low DNA-binding affinity and weak inhibition of RNA synthesis anti-tumour activity, suggest that the daunosamine ring may not be unique in conferring these properties. Any suitable side-chain at C-7 with a protonated amino group attached appropriately, could fulfil the requirements of the DNA-binding model. For maximum effect, it is desirable that such a side-chain be locked in a rigid conformation so as to mimic the natural C-7 sugar substituent, with the amino group in the same position relative to the chromophore as the natural one.

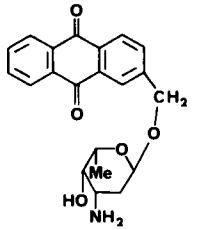
23

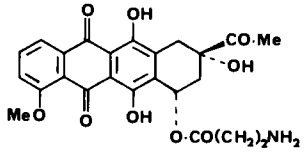


24



25





26

It has already been mentioned that *N*-acylation of the amino-sugar of daunomycin has a profound effect on DNA binding. In general, this decrease in binding ability is accompanied by a marked decrease or total loss of anti-tumour activity. However, there are some exceptions to this rule [218]. Whereas *N*-acetyldaunomycin is inactive against a L1210 test system, high dose levels in the P388 system result in a surprisingly high efficacy, possibly suggesting that a metabolic deacylation takes place in the latter at high dose levels. The properties of the analogous *N*-trifluoroacetyladriamycin 14-valerate (AD32) have been extensively explored [238,256,279—281]. It has therapeutic superiority to adriamycin, though at dose levels ten times higher; it does not appear to produce the toxicity effects of its parent compound. AD32 does not bind to DNA, and does not become localised in cell nuclei; moreover it is not metabolised to adriamycin. Clearly the modes of action of the two drugs are very different, although they appear to produce similar effects on DNA synthesis [280].

OTHER BIOLOGICAL ASPECTS

In view of the discussion in earlier sections on relationship between intercalating agents and mutagenesis, the finding that daunomycin and adriamycin evoke positive responses in the *Salmonella*/microsome test [282] is not surprising. Both drugs are themselves carcinogens, which raises questions concerning increased risk to patients being treated with them (and other anti-cancer drugs [283]). As yet, insufficient statistical data are available to answer these. It may well be significant in the context of the AD32 derivative that *N*-methylated daunomycins are non-mutagenic [284]; the intact amino group is essential for mutagenesis.

The slight superiority of daunomycin over adriamycin *in vivo* in terms of potency may well be due to differences in membrane transport properties [223,285], although their, as yet, fully unresolved metabolic pathways [285—287] may be more important.

The origins of the clinically highly significant cardiotoxicity [288] remain obscure, although many suggestions have been put forward [215], from inhibition of mitochondrial enzymes [289] to involvement in calcium regulation of cardiac muscle [290].

CONCLUDING REMARKS

Considerations of the DNA-binding abilities of many of the drugs discussed in this review, together with, in some instances, tentative molecular models of the intercalative interactions, have often led to correlations with biological action. It is perhaps surprising that these correlations are as good as indeed they are, since the action of these drugs must depend on so many factors other than just template binding. This is encouraging many to believe that further study and use of the intercalation concept may yet lead to real advances in systematic drug design, especially when taken in conjunction with an increased understanding of such factors as sequence preference in binding, cellular DNA structure and organisation, relative drug membrane transport properties, and drug metabolic pathways.

ACKNOWLEDGEMENTS

I am grateful to many colleagues for helpful discussions, especially my collaborators in Philadelphia and London. Thanks are due to those who

supplied data in advance of publication. Our work has received generous support from the Cancer Research Campaign, the International Union Against Cancer, and Roche Products Ltd.

REFERENCES

1. E.F. Gale, E. Cundliffe, P.E. Reynolds, M.H. Richmond and M.J. Waring, The Molecular Basis of Antibiotic Action (John Wiley, London, 1972).
2. J.W. Corcoran and F.E. Hahn (eds.), Antibiotics III. Mechanism of Action of Antimicrobial and Antitumour Agents (Springer-Verlag, Berlin, 1974).
3. J.W. Drake and R.H. Baltz, Annu. Rev. Biochem., 45, (1976) 11.
4. C. Heidelberger, Annu. Rev. Biochem. 43 (1974) 79.
5. T.A. Connors, FEBS Lett., 57 (1975) 223.
6. J.D. Watson and F.H.C. Crick, Nature (London), 171 (1953) 737.
7. M.H.F. Wilkins, A.R. Stokes and H.R. Wilson, Nature (London), 171 (1953) 738.
8. S. Arnott, in Proceedings of the First Cleveland Symposium on Macromolecules, ed. A.G. Walton (Elsevier, Amsterdam, 1977) pp. 87—104.
9. S. Arnott and D.W.L. Hukins, Biochem. Biophys. Res. Commun., 47 (1972) 1504.
10. S. Arnott and D.W.L. Hukins, J. Mol. Biol., 81 (1973) 93.
11. S. Arnott, R. Chandrasekaran, D.W.L. Hukins, P.J.C. Smith and L. Watts, J. Mol. Biol., 88 (1974) 523.
12. S. Arnott, D.W.L. Hukins and S.D. Dover, Biochem. Biophys. Res. Commun. 48 (1972) 1392.
13. A. Albert, The Acridines (Edward Arnold, London, 1966).
14. See, for example, G.K. McMaster and G.G. Carmichael, Proc. Nat. Acad. Sci. U.S.A., 74 (1977) 4835, for a recent application of acridines orange staining.
15. L.S. Lerman, J. Mol. Biol., 3 (1961) 18.
16. V. Luzzati, F. Masson and L.S. Lerman, J. Mol. Biol., 3 (1961) 634.
17. L.S. Lerman, Proc. Nat. Acad. Sci. U.S.A., 49 (1963) 94.
18. L.S. Lerman, J. Mol. Biol., 10 (1964) 367.
19. L.S. Lerman, J. Cell Comp. Physiol., 64 (1964) 1.
20. M.J. Waring, Nature (London), 219 (1968) 1320.
21. A.R. Peacocke and J.N.H. Skerrett, Trans. Faraday Soc., 52 (1956) 261.
22. A. Blake and A.R. Peacocke, Biopolymers, 6 (1968) 1225.
23. A.R. Peacocke, in The Acridines, ed. R.M. Acheson (John Wiley, London, 1973) pp. 723-757.
24. D.F. Bradley and M.K. Wolf, Proc. Nat. Acad. Sci. U.S.A., 45 (1959) 944.
25. D.H. Turner, G.W. Flynn, S.K. Lundberg, L.D. Faller and N. Sutin, Nature (London), 239 (1972) 215.
26. S. Neidle and T.A. Jones, Nature (London), 253 (1975) 284.
27. H.J. Li and D.M. Crothers, Biopolymers, 8 (1969) 217.
28. F. Quadrifoglio, V. Crescenzi and V. Giancotti, Biophys. Chem., 1 (1974) 319.
29. W.C. Galley and R.M. Purkey, Proc. Nat. Acad. Sci. U.S.A., 69 (1972) 2198.
30. J. Cairns, Cold Spring Harbor Symp. Quant. Biol., 27 (1962) 311.
31. See, for example; P.J. Bond, R. Langridge, K.W. Jennette and S.J. Lippard, Proc. Nat. Acad. Sci. U.S.A., 72 (1975) 4825.

32. G. Cohen and H. Eisenberg, Biopolymers, 8 (1969) 45.
33. M.J. Waring, J. Mol. Biol., 54 (1970) 247.
34. W. Fuller and M.J. Waring, Ber. Bunsenges. Phys. Chem., 68 (1964) 805.
35. J.C. Wang, J. Mol. Biol., 89 (1974) 783.
36. J.M. Saucier, B. Festy and J.B. Le Pecq, Biochimie, 53 (1971) 973.
37. J. Paoletti and J.B. Le Pecq, Biochimie, 53 (1971) 969.
38. J. Paoletti and J.B. Le Pecq, J. Mol. Biol., 59 (1971) 43.
39. W.J. Pigram, W. Fuller and M.E. Davies, J. Mol. Biol., 80 (1973) 361.
40. M.J. Waring and S.M. Henley, Nucleic Acids Res., 2 (1975) 567.
41. H.J. Li and D.M. Crothers, J. Mol. Biol., 39 (1969) 461.
42. M. Sakoda, K. Hiromi and K. Akasaka, Biopolymers, 10 (1971) 1003.
43. R.K. Tubbs, W.E. Ditmars, Jr, and Q. Van Winkle, J. Mol. Biol., 9 (1964) 545.
44. L.M. Chan and Q. Van Winkle, J. Mol. Biol., 40 (1969) 491.
45. J.C. Thomes, G. Weill and M. Daune, Biopolymers, 8 (1969) 647.
46. J. Ramstein, M. Dourlent and M. Leng, Biochem. Biophys. Res. Commun., 47 (1972) 874.
47. J. Ramstein and M. Leng, Biophys. Chem., 3 (1975) 234.
48. D.J. Patel and L.L. Canuel, Proc. Nat. Acad. Sci. U.S.A., 74 (1977) 2624.
49. D.J. Patel, Biopolymers, 16 (1977) 2739.
50. M. Zama and S. Ichimura, Biopolymers, 15 (1976) 1693.
51. V. Deubel and M. Leng, Biochimie, 56 (1974) 641.
52. D.E.V. Schmechel and D.M. Crothers, Biopolymers, 10 (1971) 465.
53. C. Steenbergen and S.C. Mohr, Biopolymers, 12 (1973) 791.
54. M. Dourlant and C. Helene, Eur. J. Biochem., 23 (1971) 86.
55. I.B. Weinstein and I.H. Finkelstein, J. Biol. Chem., 242 (1967) 3757.
56. G.G. Hammes and C.D. Hubbard, J. Phys. Chem., 70 (1966) 2889.
57. D.G. Dalgleish, H. Fujita and A.R. Peacocke, Biopolymers, 8 (1969) 633.
58. D.G. Dalgleish, A.R. Peacocke, G. Fey and C. Harvey, Biopolymers, 10 (1971) 1853.
59. D.G. Dalgleish, A.R. Peacocke, R.M. Acheson and C.W.C. Harvey, Biopolymers, 11 (1972) 2389.
60. G. Löber and G. Achtert, Biopolymers, 8 (1969) 595.
61. Y. Kubota, Y. Eguchi, K. Hashimoto, M. Wakita, Y. Honda and Y. Fujisaki, Bull. Chem. Soc., Japan, 49 (1976) 2424.
62. D.G. Dalgleish, M.C. Feil and A.R. Peacocke, Biopolymers, 11 (1972) 2415.
63. W. Müller, D.M. Crothers and M.J. Waring, Eur. J. Biochem., 39 (1973) 223.
64. R.L. O'Brien, J.G. Olenick and F.E. Hahn, Proc. Nat. Acad. Sci. U.S.A., 55 (1966) 1511.
65. J. Ciak and F.E. Hahn, Science, 156 (1967) 655.
66. T.W. Plumbridge and J.R. Brown, Biochim. Biophys. Acta., 479 (1977) 441.
67. W. Müller and D.M. Crothers, Eur. J. Biochem., 54 (1975) 267.
68. W. Müller, H. Bünemann and N. Dattagupta, Eur. J. Biochem., 54 (1975) 279.
69. A.D. Wolfe, in: Antibiotics III. Mechanism of Action of Anti-microbial and Antitumour Agents, eds. Y.W. Corcoran and F.E. Hahn (Springer-Verlag, Berlin, 1974) pp. 203-233.
70. J. Hurwitz, J.J. Furth, M. Malamy and M. Alexander, Proc. Nat. Acad. Sci. U.S.A., 48 (1962) 1222.
71. I.B. Weinstein, D. Grunberger, S. Fujimura and L.M. Fink, Cancer Res., 31 (1971) 651.
72. J.W. Drake, The Molecular Basis of Mutation (Holden-Day, San Francisco, 1970).
73. J.R. Roth, Annu. Rev. Genetics, 8 (1974) 319.

74. J.W. Drake and R.H. Baltz, Annu. Rev. Biochem., 45 (1976) 11.
75. F.H.C. Crick, L. Barnett, S. Brenner and R.J. Watts-Tobin, Nature (London), 192 (1961) 1227.
76. S. Brenner, L. Barnett, F.H.C. Crick and A. Orgel, J. Mol. Biol., 3 (1961) 121.
77. J.W. Drake, J. Cell. Comp. Physiol., 64 (1964) 19.
78. G. Streisinger, Y. Okada, J. Emrich, J. Newton, A. Tsugita, E. Terzaghi and M. Inouye, Cold Spring Harbor Symp. Quant. Biol., 31 (1966) 77.
79. J. Yourno and T. Kohno, Science, 175 (1972) 650.
80. K. Isono and J. Yourno, Proc. Nat. Acad. Sci. U.S.A., 71 (1974) 1612.
81. J.P. Schreiber and M.P. Daune, J. Mol. Biol., 83 (1974) 487.
82. J. McCann, E. Choi, E. Yamasaki and B.N. Ames, Proc. Nat. Acad. Sci. U.S.A., 72 (1975) 5135.
83. J. McCann and B.N. Ames, Proc. Nat. Acad. Sci. U.S.A., 73 (1976) 950.
84. E.C. Miller and J.A. Miller in: Chemical Mutagens: Principles and Methods for their Detection, ed. A. Hollander (Plenum, New York, 1971) Vol. 1, pp. 83-119.
85. J. Konopa, K. Koldej and J.W. Pawlak, Chem. Biol. Interactions, 13 (1976) 99.
86. B.F. Cain, G.J. Atwell and W.A. Denny, J. Med. Chem., 19 (1976) 772.
87. M.J. Waring, Eur. J. Cancer, 12 (1976) 995.
88. M.J. Waring, in: Antibiotics III. Mechanism of Action of Anti-microbial and antitumour Agents, eds. Y.W. Corcoran and F.E. Hahn (Springer-Verlag, Berlin, 1974) pp. 141-165.
89. B.A. Newton, J. Gen. Microbiology, 17 (1957) 718.
90. W.H. Elliot, Biochem. J., 86 (1963) 562.
91. J. Kock and H. von Pfeil, FEBS Lett., 18 (1971) 172.
92. E. Zylber, C. Vesco and S. Penman, J. Mol. Biol., 44, (1969) 195.
93. P.R. Cook and I.A. Brazell, Eur. J. Biochem., 84 (1978) 465.
94. M.J. Waring, J. Mol. Biol., 13 (1965) 269.
95. J.L. Bresloff and D.M. Crothers, J. Mol. Biol., 95 (1975) 103.
96. J.B. Le Pecq and C. Paoletti, J. Mol. Biol., 27 (1967) 87.
97. D.J. Patel and L.L. Canuel, Biopolymers, 16 (1977) 857.
98. M.J. Waring, Biochim. Biophys. Acta, 114 (1966) 234.
99. M.J. Waring, Biochem, J., 143 (1974) 483.
100. P. Wahl, D. Genest and J.L. Tichadou, Biophys. Chem., 6 (1977) 311.
101. J.L. Tichadou, G. Genest, P. Wahl and G. Aubel-Sadron, Biophys. Chem., 3 (1975) 142.
102. C.R. Jones and D.R. Kearns, Biochemistry, 14 (1975) 2660.
103. C.R. Jones, P.H. Bolton and D.R. Kearns, Biochemistry, 17 (1978) 601.
104. M. Liebman, J. Rubin and M. Sundaralingam, Proc. Nat. Acad. Sci. U.S.A., 74 (1977) 4821.
105. T.R. Krugh, F.N. Wittlen and S.P. Cramer, Biopolymers, 13 (1974) 2003.
106. T.R. Krugh and C.G. Reinhardt, J. Mol. Biol., 97 (1975) 133.
107. M.W. Davidson, B.G. Griggs, I.G. Lopp and W.D. Wilson, Biochim. Biophys. Acta. 479 (1977) 378.
108. D.J. Patel and L.L. Canuel, Proc. Nat. Acad. Sci. U.S.A., 73 (1976) 3343.
109. L.P.G. Wakelin and M.J. Waring, Mol. Pharmacol., 10 (1974) 544.
110. G. Woolfe, Br. J. Pharmacol. Chemother., 11 (1956) 330.
111. U. Hollstein, Chem. Rev., 74 (1974) 625.
112. W.B. Pratt, Fundamentals of Chemotherapy (Oxford, New York, 1973).
113. E. Reich and I.H. Goldberg, Prof. Nucleic Acid Res. Mol. Biol., 3 (1964) 183.

114. I.H. Goldberg, M. Rabinowitz and E. Reich, Proc. Nat. Acad. Sci. U.S.A., 48 (1962) 2094.
115. W. Müller and D.M. Crothers, J. Mol. Biol., 35 (1968) 251.
116. W. Kersten, Biochem. Biophys. Acta. 47 (1961) 610.
117. R.D.Wells and J.E. Larson, J. Mol. Biol., 49 (1970) 319.
118. L.D. Hamilton, W. Fuller and E. Reich, Nature (London), 198 (1963) 538.
119. G.V. Gursky, Mol. Biol. U.S.S.R., 3 (1970), 592.
120. H.M. Sobell, S.C. Jain, T.D. Sakore and C.E. Nordman, Nature (London), 231 (1971) 200.
121. S.C. Jain and H.M. Sobell, J. Mol. Biol., 68 (1972) 1.
122. H.M. Sobell and S.C. Jain, J. Mol. Biol., 68 (1972) 21.
123. H.M. Sobell, Progr. Nucleic Acid Res. Mol. Biol., 13 (1973) 153.
124. T.R. Krugh and Y.-C. Chen, Biochemistry, 14 (1975) 4912.
125. Y.-C.C. Chiao and T.R. Krugh, Biochemistry, 16 (1977) 747.
126. D.J. Patel, Biochim. Biophys. Acta, 442 (1976) 98.
127. D.J. Patel, Biochemistry, 13 (1974) 2396.
128. H.M. Sobell, Proc. Nat. Acad. Sci. U.S.A., 69 (1972) 2483.
129. H.M. Sobell, Proc. Nat. Acad. Sci. U.S.A., 72 (1975) 279.
130. F.S. Allen, R.P. Moen and U. Hollstein, J. Am. Chem. Soc., 98 (1976) 864.
131. F.S. Allen, M.B. Jones and U. Hollstein, Biophys. J., 20 (1977) 69.
132. E. Hirschberg, in: Antibiotics III. Mechanism of Action of Anti-microbial and antitumour Agents, eds. Y.W. Corcoran and F.E. Hahn (Springer-Verlag, Berlin, 1974) pp. 274-303.
133. D. Rosi, G. Peruzzotti, E.W. Dennis, D.A. Berberian, H. Frede, B.F. Tullar and S. Archer, J. Med. Pharm. Chem., 10 (1967) 867.
134. P.E. Hartman, K. Levine, Z. Hartman and H. Berger, Science, 172, (1971) 1058.
135. W.F. Benedict, M.S. Baker, L. Haroun, E. Choi and B.N. Ames, Cancer Res., 37 (1977) 2209.
136. P.B. Hulbert, E. Bueding and P.E. Hartman, Science, 186 (1974) 647.
137. S.M. Sieber, J. Whang-Peng, D.G. Johns and R.H. Adamson, Biochem. Pharmacol., 22 (1973) 1253.
138. R. Bases, Cancer Res., 30 (1970) 2007.
139. I.B. Weinstein, R. Chernoff, I. Finkelstein and E. Hirchberg, Mol. Pharmacol., 1 (1965) 297.
140. I.B. Weinstein, R. Carchman, E. Marner and E. Hirschberg, Biochim. Biophys. Acta, 142 (1967) 440.
141. I.B. Weinstein and E. Hirschberg, Prog. Mol. Subcell. Biol., 2 (1971) 232.
142. R.A. Carchman, E. Hirschberg and I.B. Weinstein, Biochim. Biophys. Acta, 179 (1969) 158.
143. E. Hirschberg, I.B. Weinstein, N. Gersten, E. Marner, T. Finkelstein and R. Carchman, Cancer Res., 28 (1968) 601.
144. S. Neidle, Biochim. Biophys. Acta, 454 (1976) 207.
145. C.H. Wei and J.R. Einstein, Acta Crystallogr., B34 (1978) 205.
146. M.L. Post, O. Kennard and A.S. Horn, Nature (London), 256 (1975) 342.
147. M.J. Heller, A.T. Tu and G.E. Maciel, Biochemistry, 13 (1974) 1623.
148. M.J. Waring, J. Pharmacol. Exp. Ther., 186 (1973) 385.
149. W.A. Denny and B.F. Cain, J. Med. Chem., 21 (1978) 430.
150. R. Zilversmit, Mol. Pharmacol., 7 (1971) 674.

151. R. Zilversmit, Mol. Pharmacol., 6 (1970) 172.
152. R. Bases, A. Leifer, H. Rozycki, C. Blake, Jr and S. Neubort, Cancer Res., 37 (1977) 2177.
153. R. Bases, F. Mendez, F. Elequin, D. Lieberskind, A. Kozin and S. Neubort, Cancer Res., 38 (1978) 781.
154. J. Hartwell and B. Abbott, Adv. Chemother. Pharmacol., 7 (1969) 117.
155. G.H. Svoboda, G.A. Poore and M.L. Montfort, J. Pharm. Sci., 57 (1968) 1720.
156. B.K. Bhuyan, T.J. Fraser and L.H. Li, Cancer Res., 32 (1972) 2538.
157. K.W. Kohn, M.J. Waring, D. Glaubiger and C.A. Friedman, Cancer Res., 35 (1975) 71.
158. J.B. Le Pecq, N. Dat-Xuong, C. Gosse and C. Paoletti, Proc. Nat. Acad. Sci. U.S.A., 71 (1974) 5078.
159. J.B. Le Pecq, M. Le Bret, C. Gosse, C. Paoletti and N. Dat-Xuong, Jerusalem Symp. Quantum Chem. Biochem., 7 (1974) 501.
160. P. Lecointe, P. Lesca, S. Cros and C. Paoletti, Chem., Biol. Interactions, 20 (1978) 113.
161. P.B. Dervan and M.M Becker, J. Am. Chem. Soc., 100 (1978) 1968.
162. M.J. Waring, in: Drug Action at the Molecular Level, ed. G.C.K. Roberts (MacMillan, London, 1977) pp. 167-189.
163. M.J. Waring, L.P.G. Wakelin and J.S. Lee, Biochim. Biophys. Acta, 407 (1975) 200.
164. M.J. Waring and L.P.G. Wakelin, Nature (London), 252 (1974) 653.
165. L.P.G. Wakelin and M.J. Waring, Biochem. J., 157 (1976) 721.
166. G. Ughetto and M.J. Waring, Mol. Pharmacol., 13 (1977) 579.
167. D.M. Crothers, Biopolymers, 6 (1968) 575.
168. P. Costantino, P. De Santis, G. Ughetto and M.J. Waring, FEBS Lett., 88 (1978) 349.
169. C. Courseille, F. Leroy, M. Hospital and J. Barbet, Acta Crystallogr. B33 (1977) 1565.
170. C. Courseille, F. Leroy, B. Busetta and J. Barbet, Acta Crystallogr., B33 (1977) 1570.
171. J.B. Le Pecq, M. Le Bret, J. Barbet and B. Roques, Proc. Nat. Acad. Sci. U.S.A., 72 (1975) 2915.
172. J. Barbet, B.P. Roques, S. Combrisson and J.B. Le Pecq, Biochemistry. 15 (1976) 2642.
173. E.S. Canellakis, Y.H. Shaw, W.E. Hanners and R.A. Schwartz, Biochim. Biophys. Acta, 418 (1976) 277.
174. E.S. Canellakis and R.A. Bellantone, Biochim. Biophys. Acta, 418 (1976) 290.
175. E.S. Canellakis, V. Bono, R.A. Bellantone, J.S. Krakow, R.M. Fico and R.A. Schulz, Biochim. Biophys. Acta, 418 (1976) 300.
176. R.M. Fico, T.K. Chen and E.S. Canellakis, Science, 198 (1977) 53.
177. L.P.G. Wakelin, M. Romanos, E.S. Canellakis and M.J. Waring, Studia Biophys., 60 (1976) 111.
178. J.G. Andrew, L.P.G. Wakelin and M.J. Waring, unpublished results.
179. P.J.C. Smith and S. Arnott, Acta Crystallogr., A34 (1978) 3.
180. H.R. Wilson, Diffraction of X-rays by Proteins, Nucleic Acids and Viruses (Edward Arnold, London, 1966).
181. D.M. Neville, Jr. and D.R. Davies, J. Mol. Biol., 17 (1966) 57.
182. C.J. Alden and S. Arnott, Nucleic Acids Res., 2 (1975) 1701.
183. C.J. Alden and S. Arnott, Nucleic Acids Res., 4 (1977) 3855.
184. S. Arnott, W. Fuller, A. Hodgson and I. Prutton, Nature (London), 220 (1968) 561.
185. N.J. Pritchard, A. Blake and A.R. Peacocke, Nature (London), 212 (1966) 360.
186. J.P. Glusker and K.N. Trueblood, Crystal Structure Analysis: a Primer (Oxford, New York, 1972).
187. B.R. Reid, N.S. Ribeiro, G. Gould, G.T. Robillard, C.W. Hilbers and R.G. Shulman, Proc. Nat. Acad. Sci. U.S.A., 72 (1975) 2049.

188. N.C. Seeman, R.O. Day and A. Rich, Nature, 253 (1975) 324.
189. S. Neidle, G.L. Taylor, M.R. Sanderson, H.M. Berman and H.-S. Chieh, Nucleic Acids Res., 5 (1978) 4417.
190 C.-C. Tsai, S.C.-Jain and H.M. Sobell, Proc. Nat. Acad. Sci. U.S.A., 72 (1975) 628.
191. C.-C. Tsai, S.C. Jain and H.M. Sobell, Phil. Trans. R. Soc. Lond. B272 (1975) 137.
192. C.-C. Tsai, S.C. Jain and H.M. Sobell, J. Mol. Biol., 114 (1977) 301.
193. S.C. Jain, C.-C. Tsai and H.M. Sobell, J. Mol. Biol., 114 (1977) 317.
194. T.D. Sakore, S.C. Jain, C.-C. Tsai and H.M. Sobell, Proc. Nat. Acad. Sci. U.S.A., 74 (1977) 188.
195. H.M. Sobell, S.C. Jain, T.D. Sakore, B.S. Reddy, K.K. Bhandary and T.P. Seshadri, in: Symposium Volume, International Symposium on Biomolecular Structure, Conformation, Function and Evolution (Pergamon Press 1978), in the press.
196. T.P. Seshadri, T.D. Sakore and H.M. Sobell, Abstracts Am. Crystallogr. Assoc., 5 (1977) 84.
197. T.P. Seshadri, T.D. Sakore, B.S. Reddy and H.M. Sobell, Abstracts Am. Crystallogr. Assoc., 6 (1978) 12.
198. D.J. Patel and C. Shen, Proc. Nat. Acad. Sci. U.S.A., 75 (1978) 2553.
199. S. Neidle, A. Achari, G.L. Taylor, H.M. Berman, H.L. Carrell, J.P. Glusker and W.C. Stallings, Nature (London), 269 (1977) 304.
200. H.M. Sobell, C.-C. Tsai, S.C. Jain and S.G. Gilbert, J. Mol. Biol., 114 (1977) 333.
201. H.M. Berman, S. Neidle and R.K. Stodola, Proc. Nat. Acad. Sci. U.S.A., 75 (1978) 828.
202. H.M. Sobell, C.-C. Tsai, S.G. Gilbert, S.C. Jain and T.D. Sakore, Proc. Nat. Acad. Sci. U.S.A., 73 (1976) 3068.
203. H.M. Berman, and S. Neidle, in: Stereodynamics of Molecular Systems, ed. R.H. Sarma (Pergamon, New York, 1979), in the press.
204. J.M. Rosenberg, N.C. Seeman, R.O. Day and A. Rich, J. Mol. Biol., 104 (1976) 145.
205. B. Hingerty, E. Subramanian, S. Stellman, T. Sato, S. Broyde and R. Langridge, Acta Crystallogr., B32 (1976) 2998.
206. N.C. Seeman, J.M. Rosenberg, F.L. Suddath, J.J.P. Kim and A. Rich, J. Mol. Biol., 104 (1976) 109.
207. M.A. Viswamitra, O. Kennard, P.G. Jones, G.M. Sheldrick, S. Salisbury, L. Falvello and Z. Shakked, Nature (London), 273 (1978) 687.
208. G. Felsenfeld, Nature (London), 271 (1978) 115.
209. M. Levitt, Proc. Nat. Acad. Sci. U.S.A., 75 (1978) 640.
210. J.L. Sussman and E.N. Trifonov, Proc. Nat. Acad. Sci. U.S.A., 75 (1978) 103.
210a. J.R. Brown, Prog. Med. Chem., 15 (1978) 126.
211. A. Di Marco, F. Arcamone and F. Zunino, in Ref. 2, pp. 101-128.
212. A. Di Marco and F. Arcamone, Arzneim.-Forsch., 25 (1975) 368.
213. A. Di Marco, Cancer Chemother. Rep., 6 (1975) 91.
214. S.K. Carter, Cancer Chemother. Rep., 6 (1975) 389.
215. S. Neidle, in: Topics in Antibiotic Chemistry, Vol. 2, ed. P.G. Sammes (Ellis Horwood, Chichester, 1978), pp. 240–278.
216. I.H. Goldberg, T.A. Beerman and R. Poon, in: Cancer, Vol. 5, ed. F.K. Becker (Plenum, New York, 1977) pp. 427-456.
217. H.S. Schwartz, Biomedicine, 24 (1976) 317.
218. D.W. Henry, in: Cancer Chemotherapy, ed. A.C. Sartorelli, (Am. Chem. Soc. Symp. Ser.) 30 (1976) 15.
219. F. Arcamore, Lloydia, 40 (1977) 45.
220. Y. Daskal, C. Woodard, S.T. Crooke and H. Busch, Cancer Res., 38 (1978) 467.

221. A. Di Marco, in: Handbook of Experimental Pharmacology, Vol. 38, eds. A.C. Sartorelli and D.G. Johns (Springer-Verlag, Berlin, 1975) pp. 593-614.
222. G. Noël, C. Peterson, A. Trouet and P. Tulkens, Eur. J. Cancer, 14 (1978) 363.
223. T. Skovsgaard, Biochem. Pharmacol., 26 (1977) 215.
224. F. Zunino, A. Di Marco, A. Zaccara and G. Luoni, Chem. Biol. Interactions, 9 (1974) 25.
225. W.D. Meriwether and N.R. Bachur, Cancer Res., 32 (1972) 1137.
226. V. Barthelemy-Clavey, C. Molinier, G. Aubel-Sadron and R. Maral, Eur. J. Biochem., 69 (1976) 23.
227. D.C. Ward, E. Reich and I.H. Goldberg, Science, 149 (1965) 1259.
228. R. Silvestrini, L. Lenaz, G. Di Fronzo and O. Sanfilippo, Cancer Res., 33 (1973) 2954.
229. S.T. Crooke, V.H. Duvernay, L. Galvan and A.W. Prestayko, Mol. Pharmacol., 14 (1978) 290.
230. F. Zunino, R. Gambetta, A. Di Marco, A. Zaccara and G. Luoni, Cancer Res., 35 (1975) 754.
231. M.F. Goodman, G.M. Lee and N.R. Bachur, J. Biol. Chem., 252 (1977) 2670.
232. T. Facchinetti, A. Mantovani, L. Cantoni, R. Cantoni and M. Salmona, Chem. Biol. Interactions, 20 (1978) 97.
233. P. Chandra, F. Zunino, A. Götz, D. Gericke, R. Thorebeck and A. Di Marco, FEBS Lett., 21 (1972) 264.
234. A. Di Marco, A.M. Casazza, R. Gambetta, R. Supino and F. Zunino, Cancer Res., 36 (1976) 1962.
235. F. Arcamone, S. Penco, A. Vigevani, S. Redaelli, G. Franchi, A. Di Marco, A.M. Casazza, T. Dasdia, F. Formelli, A. Necco and C. Soranzo, J. Med. Chem., 18 (1975) 703.
236. F. Arcamone, L. Bernardi, P. Giardino, B. Patelli, A. Di Marco, A.M. Casazza, G. Pratesi and P. Reggiani, Cancer Treatment Rep., 60 (1976) 829.
237. T. Facchinetti, A. Mantovani, R. Cantoni, L. Cantoni, C. Pantorotto and M. Salmona, Biochem. Pharmacol., 26 (1977) 1953.
238. M. Israel, E.J. Modest and E. Frei, Cancer Res., 35 (1975) 1365.
239. E. Calendi, A. Di Marco, M. Reggiani, B. Scarpinato and L. Valentini, Biochim. Biophys. Acta, 103 (1965) 25.
240. F. Zunino, R. Gambetta, A. Di Marco and A. Zaccara, Biochim. Biophys. Acta, 277 (1972) 489.
241. A. Di Marco, F. Zunino, R. Silvestrini, C. Gambaruccu and R.A. Gambetta, Biochem. Pharmacol., 20 (1971) 1323.
242. F. Zunino, R. Gambetta, A. Di Marco, G. Luoni and A. Zaccara, Biochem. Biophys. Res. Commun., 69 (1976) 744.
243. K.C. Tsou and K.F. Yip, Cancer Res., 36 (1976) 3367.
244. D.G. Dalgeish, G. Fey and W. Kersten, Biopolymers, 13 (1974) 1757.
245. E.J. Gabbay, D. Grier, R.E. Fingerle, R. Reimer, R. Levy, S.W. Pearce and W.D. Wilson, Biochemistry, 15 (1976) 2062.
246. T.R. Krugh and M.A. Young, Nature (London), 269 (1977) 627.
247. W. Kersten, H. Kersten and W. Szybalski, Biochemistry, 5 (1966) 236.
248. Y.M. Huang and D.R. Phillips, Biophys. Chem., 6 (1977) 363.
249. M.J. Waring, in: Topics in Infections Diseases, Vol. 1, eds. J. Drews and F.E. Hahn (Springer-Verlag, Vienna, 1974) pp. 77-90.
250. T.W. Plumbridge and J.R. Brown, Biochim. Biophys. Acta, 479 (1977) 441.

251. S.R. Byrn and G.D. Dolch, J. Pharm. Sci., 67 (1978) 688.
252. F. Zunino, A. Di Marco and A. Velcich, Cancer Lett., 3 (1977) 271.
253. A. Di Marco, A.M. Casazza, T. Dasdia, A. Necco, G. Pratesi, P. Rivolta, A. Velcich, A. Zaccara and F. Zunino, Chem. Biol. Interactions, 19 (1977) 291.
254. V. Barthelemy-Clavey, J.C. Maurizot and P.J. Sicard, Biochimie, 55 (1973) 859.
255. T.H. Smith, A.N. Fujiwara and D.W. Henry, J. Med. Chem., 21 (1978) 280.
256. S.K. Sengupta, R. Seshadri, E.J. Modest and M. Israel, Abstr. Am. Assoc. Cancer Res., 17 (1976) 109.
257. D.R. Phillips, A. Di Marco and F. Zunino, Eur. J. Biochem., 85 (1978) 487.
258. J. Doskocil and I. Fric, FEBS Lett., 37 (1973) 55.
259. R.H. Shafer, Biochem. Pharmacol., 26 (1977) 1729.
260. R.L. Momparler, M. Karon, S.E. Siegel and F. Avila, Cancer Res., 36 (1976) 2891.
261. R. Anguili, E. Foresti, L. Riva di Sanseverino, N.W. Isaacs, O. Kennard, W.D.S. Motherwell, D.L. Wampler and F. Arcamone, Nature New Biol., 234 (1971) 78.
262. M.C. Wani, H.L. Taylor, M.E. Wall, A.T. McPhail and K.D. Onan, J. Am. Chem. Soc., 97 (1975) 5955.
263. G.R. Pettit, J.J. Einck, C.L. Herald, R.H. Ode, R.B. Von Dreele, P. Brown, M.G. Brazhnikova and G.F. Gause, J. Am. Chem. Soc., 97 (1975) 7387.
264. R.B. Von Dreele and J.J. Einck, Acta Crystallogr., B33 (1977) 3283.
265. S. Neidle and G. Taylor, Biochim Biophys. Acta, 479 (1977) 450.
266. C. Courseille, B. Busetta, S. Geoffre and M. Hospital, private communication.
267. S. Neidle, to be published.
268. W.J. Pigram, W. Fuller and L.D. Hamilton, Nature New Biol., 235 (1972) 17.
269. S. Neidle, Cancer Treatment Rep., 61 (1977) 928.
270. J.C. Double and J.R. Brown, J. Pharm. Pharmacol., 27 (1975) 502.
271. J.C. Double and J.R. Brown, J. Pharm. Pharmacol., 28 (1976) 166.
272. F. Suzuki, S. Trenbeath, R.D. Gleim and C.J. Sih, J. Am. Chem. Soc., 100 (1978) 2272.
273. S. Neidle, Nature (London), 268 (1977) 195.
274. A. Di Marco, A.M. Casazza, F. Guiliani, G. Pratesi, F. Arcamone, L. Bernadi, G. Franchi, P. Giardino, B. Patelli and S. Penco, Cancer Treatment Rep., 62 (1978) 375.
275. R. Supino, A. Necco, T. Dasdia, A.M. Casazza and A. Di Marco, Cancer Res., 37 (1977) 4523.
276. M. Israel and R. Seshadri, Abstr. Am. Chem. Soc. Meeting, 1978.
277. M.A. Apple, H. Yanagasawa and G. Subrahmanyam, Abstr. Am. Assoc. Cancer Res., 18 (1977) 406; M.A. Apple, personal communication.
278. F. Zunino, R. Gambetta, A. Di Marco, A. Velcich, A. Zaccara, F. Quadrifoglio and V. Crescenzi, Biochim. Biophys. Acta, 476 (1977) 38.
279. M. Israel, P.M. Wilkinson, W.J. Pegg and E. Frei, Cancer Res., 38 (1978) 365.
280. L.M. Parker, M. Hirst and M. Israel, Cancer Treatment Rep., 62 (1978) 119.
281. M. Israel, W.J. Pegg and P.M. Wilkinson, J. Pharmacol. Exp. Ther., 204 (1978) 696.
282. W.F. Benedict, M.S. Baker, L. Haroun, E. Choi and B.N. Ames, Cancer Res., 37 (1977) 2209.
283. S.M. Sieber Cancer Chemother. Rep., 59 (1975) 915.
284. K. Umezawa, M. Sawamura, T. Matsushima and T. Sugimura, Cancer Res., 38 (1978) 1782.
285. N.R. Bachur, in Ref. 218, p. 58.
286. H. Loveless, E. Arena, R.L. Felsted and N.R. Bachur, Cancer Res., 38 (1978) 593.

287. N. R. Bachur, M. Steele, W.D. Meriwether and R.C. Hildebrand, J. Med. Chem., 19 (1976) 651.
288. L. Lenaz and J.A. Page, Cancer Treatment Rev., 3 (1976) 111.
289. K. Folkers, M. Liu, T. Watanabe and T.H. Porter, Biochem. Biophys. Res. Commun., 77 (1977) 1536.
290. F. Villani, F. Piccinini, P. Merelli and L. Favalli, Biochem. Pharmacol., 27 (1978) 985.

This Page Intentionally Left Blank

Progress in Medicinal Chemistry — Vol. 16, edited by G.P. Ellis and G.B. West

4 Inhibitors of Enzymes of Microbial Membranes; Agents Affecting Mg^{2+}-Activated Adenosine Triphosphatase

S.M. HAMMOND, B.Sc., Ph.D.

*Department of Biological Sciences, Purdue University, W. Lafayette, Indiana 47907, U.S.A.**

*Present address: Department of Microbiology, The University of Leeds, Leeds LS2 9JT, England.

INTRODUCTION

The structure and function of the magnesium-activated adenosine triphosphatases (Mg^{2+}-ATPase) from energy-coupling membranes of chloroplasts, mitochondria and bacteria appear to be similar [1—6]. It is possible to isolate from coupling membranes a high molecular weight ATPase complex, consisting of two fractions, known as F_1 and F_0. The former is a water-soluble protein which has all the ATPase activity of the complex. In contrast, the F_0 protein requires detergent-solubilization to release it from the membrane and is devoid of ATPase activity.

A wide range of inhibitors of membrane ATPase have been described; some act by binding to the F_0-complex, while others apparently modify the F_1 region of the ATPase. In order to fully understand the mechanism of action of these inhibitors it is necessary to briefly describe the structure and function of energy-transducing membranes.

ENERGY-TRANSDUCING MEMBRANES

The energy-transducing membranes of mitochondria and bacteria are able to utilize available substrates to provide energy for the performance of work such as the accumulation of metabolites against a concentration gradient or locomotion. Two distinct but interrelated enzyme systems have been implicated in this phenomenon. The first is the membrane-bound Mg^{2+}-ATPase, the terminal enzyme of oxidative phosphorylation, which catalyzes the reaction,

$$ADP + P_i + nH^+ \rightleftarrows ATP + H_2O$$

(n is pH dependent; 0.7 at pH 7 [7])

The second involves the enzymes of the oxidative (electron-transport) chain. Oxidative capacity is not essential for active transport since under anaerobic conditions, where organisms have limited or no oxidative enzyme capacity, active solute uptake may proceed. Energy-linked transport may also occur in mitochondrial or bacterial vesicles made in such a way that ATPase activity is lost or in which the ATPase is inhibited. This suggests that active transport is not necessarily coupled directly to ATP hydrolysis and that solute translocation or flagellar motion may be driven by the 'energized state' of the membrane, designated ($\sim$), *i.e.*,

Oxidation ⇋ ~ ⇌ ATP
⥮
Work *e.g.*, active transport or flagellar motion.

The exact nature of this 'energized-state' and the mechanism by which the major bioenergetic activities of the membrane are coupled is still controversial [8—13]. Mitchell in his 'chemiosmotic' hypothesis [14] suggested a mechanism for the coupling of electron transfer to ATP synthesis, proposing that the electron transport chain is arranged in loops across the energy-transducing membrane and as a result of the transfer of two electrons to water, protons are expelled (*Figure 4.1*). The electrogenic expulsion of protons makes the interior negative with respect to the surrounding environment. The established proton gradient represents a store of free energy and because of the difference in concentration and electrical potential each expelled proton experiences a force

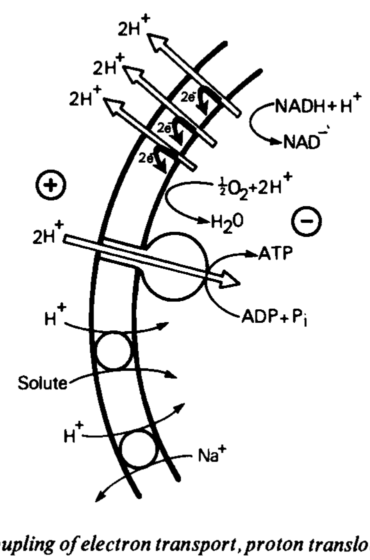


Figure 4.1. Chemiosmotic coupling of electron transport, proton translocation and metabolite uptake in bacteria and mitochondria. Electrons and protons from NADH, with protons from the internal medium, are donated to a series of carriers arranged in loops across the membrane. In mitochondria there are believed to be three such loops, in bacteria only two. The passage of electrons results in the expulsion of 2 protons per loop, i.e., 6 protons in mitochondria, 4 in bacteria. The diffusion of the protons back down the gradient into the mitochondria or bacterial cell may be used to drive ATP-synthesis by means of the proton translocating ATP synthetase, to drive solute uptake, using a proton symport or to drive solute excretion using a proton antiport [11,13,136].

(the protonmotive force) tending to draw it back across the membrane. The movement of protons back through the membrane in response to that force may be made to do work. Particles visible on the surface of energy-transducing membranes have been isolated and found to couple the diffusion of protons back through the membrane with the synthesis of ATP (*Figure 4.1*), two protons being required per molecule of ATP synthesized. The proton gradient may also be used to drive uptake or excretion of metabolites, allowing the electron transfer process to directly provide energy for active transport, without going through ATP [15,16]. The 'localized proton' hypotheses of Williams similarly requires the participation of protons in active transport and ATP synthesis, but does not specifically include a trans-membrane proton movement [10,17,18]. Indirect mechanisms for ATP synthesis have also been proposed, the 'conformational hypotheses' [19—22] suggest that changes in protein conformation may serve as the driving force in ATP production. Other proposed indirect mechanisms involve the phosphorylation of hypothetical intermediates [23,24]. Several pieces of evidence lend strong support to the 'chemiosmotic' theory of oxidative phosphorylation.

(a) There is direct correlation between the 'energization' of membranes and the generation of the proton motive force (PMF).
(b) ATP-synthesis can be driven by an artificially generated PMF.
(c) Isolated ATP-synthetase can be reassembled into membrane vesicles and is then capable of phosphorylation.
(d) The indirect mechanisms fail to provide an adequate explanation of the action of uncouplers (p. 230).

ATPases of energy-transducing membranes

The recognition that the soluble ATPase (F_1) from mitochondrial ATPase was the factor coupling substrate oxidation to ATP synthesis [25—27], and its subsequent localization as small subunits, 90 Å diameter, on the inner mitochondrial membrane has given valuable insight into the topology, structure and function of membrane bound ATPase [21—28]. It is believed that mitochondrial ATPase and the equivalent systems associated with the energy-transducing membranes of chloroplasts and bacteria [1—4] function as proton-translocating reversible ATPase (*Figure 4.1*).

It is possible to prepare submitochondrial vesicles by treating extracted mitochondria with membrane dispersing reagents, *e.g.*, Lubrol W or digitonin, or by exposure to ultrasonic radiation. The resultant vesicles have been shown to result from the pinching-off of the mitochondrial cristae and hence the membrane and its components are inverted, as compared with the parent

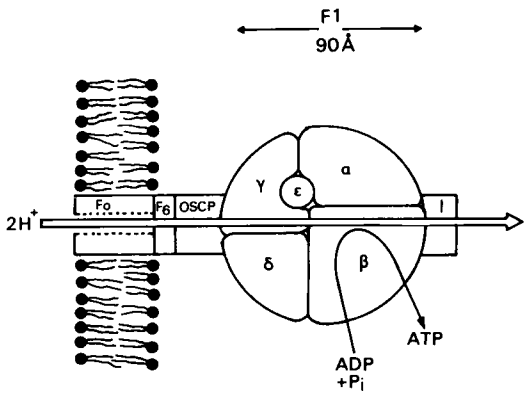


Figure 4.2. Proton-translating membrane bound Mg^{2+}-ATPase of mitochondria. The enzyme consists of 3 regions (a) the F_1, the ATPase proper, situated in projections coating the inside of the mitochondrial membrane (b) the F_6/OSCP region which serves to connect F_1 to the membrane and (c) the proton-translocating section of the enzyme, a proteolipid complex (F_0) located in the membrane. Passage of 2 protons through the proton channel facilities the formation of 1 molecule of ATP from ADP and P_i. The actual site of synthesis is probably the β-subunit.

mitochondrion. Provided these vesicles are sealed, they are capable of both electron transport and oxidative phosphorylation. With judicious use of detergent, the entire ATPase (*i.e.*, F_0/F_1 complex) may be extracted from submitochondrial vesicles or bacteria [29—32]. Incorporation of this complex into liposomes has been shown to permit ATP-dependent proton translocation concomitant with the formation of an electrochemical gradient.

The membrane bound Mg^{2+}-activated ATPase from mammalian mitochondria has received most attention. Treatment of mammalian mitochondria with urea [33], sodium bromide [34] or cardiolipin [35] released a water soluble ATPase (F_1), which has been extensively purified. *Figure 4.2* is a schematic representation of the Mg^{2+}-ATPase from mitochondrial membranes. The purified F_1 was cold-labile, dissociating into five types of protein subunit (α, β, γ, δ and ε) (*Table 4.1*) none of which had enzymic activity [1,3,23]. The active site was thought to be located in the two heaviest subunits (α and β), since trypsin treatment yielded a protein containing only these units with intact ATPase activity [36] and probably was restricted to the β subunits [37,38]. Ammonia treatment of F_1 extracted from mitochondria with urea released a polypeptide, known as the oligomycin sensitivity-conferring protein (OSCP), which has been shown to be necessary for the binding of F_1 to the membrane [39]. Another protein, designated F_6, has been removed by

Table 4.1. COMPOSITION AND SUBUNIT SIZES OF PURIFIED SOLUBLE MEMBRANE ATPases FROM MITOCHONDRIA (F_1) AND BACTERIA (BF_1)

Source	*Mol. wt.* ($X\ 10^{-3}$)	*Subunit mol. wt* ($X\ 10^{-3}$) *and composition*					*References*
		α	β	γ	δ	ε	
Rat liver mitochondria	340—380	62.5 (3)	57 (3)	36 (1)	12 (1)	7.5 (1)	84,85
Yeast mitochondria	340	58 (3)	55 (3)	38 (1)	10 (1)	8.5 (1)	49,51
E. coli	340	56 (2)	52 (2)	32 (2)	21 (1 or 2)	11.5 (2)	56,86
S. typhimurium		57 (3)	52 (3)	31 (1)	21.5 (1)	13 (1)	56
S. faecalis	385	60 (3)	55 (3)	37 (1)	20 (1)	12 (1)	72,73
B. megaterium	399	68 (3)	65 (3)				68-70

The number of each subunit type present in the complex is given in parenthesis.

treatment with silicotungstate and it is believed that this protein is directly responsible for the binding of F_1 to F_0. The final component, the ATPase inhibitor protein (I), was readily removed from F_1 by trypsin [40,41] or by Sephadex chromatography [42]. It was shown to be a low molecular weight trypsin-sensitive protein, apparently existing as a distinct entity on the mitochondrial particle [1] and it was believed to serve a regulatory function in oxidative phosphorylation controlling the back-flow of energy in ATP to ADP-driven reactions [40—48].

The part of the ATPase known as F_0 is an integral part of the mitochondrial membrane and consists of three or four hydrophobic proteins [29] with no discernable ATPase activity. The F_0 component may be extracted from the mitochondrial membrane with detergent and incorporated into liposomes. Incorporation of F_0 into liposomes markedly increased their permeability to protons, an effect which was reversed by addition of the F_1/OSCP/F_6 complex [1—4]. This suggests that F_0 may be a proton-conducting channel which may be sealed by the binding of the completely soluble ATPase.

The F_1/F_0 complex from yeast mitochondria contained at least ten distinct polypeptides [49—51], five of which (α—ε) were associated with the cold labile F_1-ATPase (*Table 4.1*) [49—53] and one was the OSCP [51] which linked the F_1 to the remaining four hydrophobic polypeptides tightly asso-

ciated with the inner mitochondrial membrane [49]. The inhibitor protein (I) of yeast mitochondria, like that of mammalian mitochondria was a discrete entity [1,54].

The membranes of bacteria possess a prominent Mg^{2+}-activated ATPase, but only a small amount of ouabain-sensitive Na^+, K^+-ATPase [55]. Mg^{2+}-ATPase complexes have been purified from *Escherichia coli* [56—60], *Micrococcus lysodeikticus* [61—64], *Mycobacterium phlei* [65] *Salmonella typhimurium* [56], *Alcaligenes faecalis* [66], *Bacillus subtilis* [67] *B. megaterium* [68—70] and *Streptococcus faecalis* [71—73].

The best studied bacterial ATPase is the Mg^{2+}-activated enzyme from the membranes of *S. faecalis* [71,74,75], although recent studies have extended to other species [2,13,76]. Electron micrograph studies have demonstrated that bacterial membranes contain particles similar in size and shape to the described ATPase of mitochondria [77—81]. The use of ferritin-labelled antibodies to purified bacterial ATPase has shown that these particles constitute the bacterial enzyme [82] and as in the case of mitochondria are located on the inner surface of the energy-coupling membrane [57,83]. Differences have been demonstrated in both the composition and subunit size in purified soluble ATPase from bacteria (BF_1) and mitochondria (F_1) (*Table 4.1*). The β subunit of BF_1 appeared to be the active site of the enzyme, the γ subunit was required for reassembly of the catalytic site after cold treatment, and the δ subunit functioned as the link between the BF_1 and BF_0 [87, 88]. Like mitochondria, the bacterial soluble ATPase (BF_1) possessed an inhibitor protein [89], (subunit ε), with a molecular weight of 10 000, but this protein unlike that of mitochondria appeared as an integral part of the enzyme complex (*Figure 4.3*). This protein, although capable of regulating

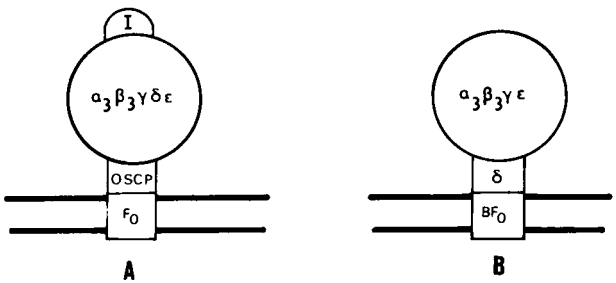


Figure 4.3. Diagrammatic comparison of (A) mitochondrial and (B) bacterial membrane-bound Mg^{2+}-ATPase (adapted from [13]).

purified bacterial Mg^{2+}-ATPase had no effect upon mitochondrial ATPases [89].

INHIBITORS OF MEMBRANE BOUND ATPases

Several agents are able to uncouple oxidative phosphorylation from ATP synthesis. Such compounds, known as uncouplers, halt ATP synthesis by a mechanism distinct from that of agents which act directly upon the ATP synthetase, the true ATPase inhibitors.

In the presence of oxidizable substrate, ADP, oxygen and inorganic phosphate, the rates of mitochondrial respiration and phosphorylation are fast (*Figure 4.4A*) and the mitochondria are in the active state [90,91]. In the controlled state, that is, in the presence of substrate and oxygen but in the absence of ADP (or phosphate), there is little respiration in tightly-coupled mitochondria, the rate of respiration being controlled by the ADP/ATP ratio. When this ratio is high, respiration is rapid but under conditions where ATP accumulates at the expense of ADP (*i.e.* when the rate of energy requirement falls below the rate of ATP synthesis) respiration rate falls. This phenomenon, known as respiratory control, is found in mitochondria but not in bacteria. The best known uncouplers include 2,4-dinitrophenol (DNP), carbonylcyanide *m*-chlorophenylhydrazone (CCCP), carbonylcyanide *p*-trifluoromethylphenylhydrazone (FCCP) and trichlorosalicylanilide, all relatively simple organic compounds. Uncouplers are lipid soluble and are believed to dissolve in biological membranes, acting as proton conductors, effectively 'short-circuiting' the proton motive force. Uncouplers, by dissipating the 'energized state' of the membrane, deprive the ATP synthetase of its energy input, effectively bringing ATP synthesis to a halt. Respiratory control is lost in the presence of uncoupler, allowing substrate oxidation to proceed at maximum rate (*Figure 4.4*, trace B) producing heat rather than ATP. Hence uncouplers, although bringing ATP synthesis to a halt, do not have a direct effect upon membrane ATPase and will not be discussed further. Several reviews of uncoupler action have been published [92—98].

It is possible to divide the true inhibitors of Mg^{2+}-ATPase into two groups. Inhibitors shown to act on both the soluble and the membrane-bound ATPase are believed to have their site of action in the F_1 or BF_1 region of the enzyme (*Figure 4.5*). Those agents which have been shown only to inhibit the enzyme while it was membrane bound, to have no effect upon the purified soluble enzyme and to reduce proton permeability in preparations devoid of F_1 or BF_1, have their primary action in the proteolipid region of the membrane *i.e.*,

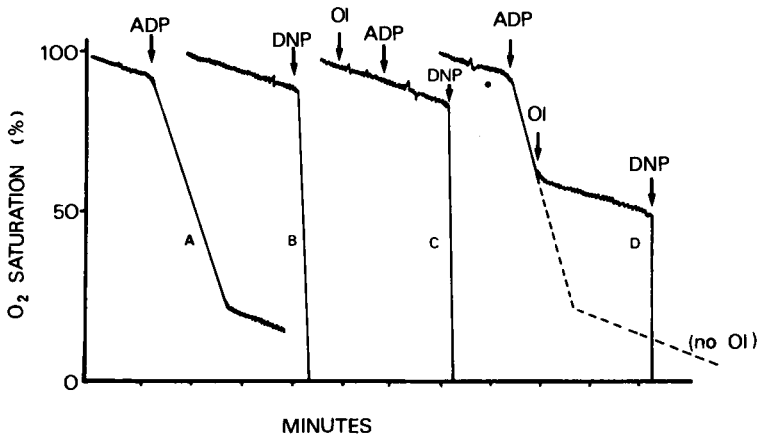


Figure 4.4. Comparison of the effects of an uncoupler, e.g., DNP and an inhibitor of Mg^{2+}-ATPase, e.g., oligomycin on the O_2-consumption of rat liver mitochondria. Oxygen consumption of freshly prepared mitochondria suspended in buffer (containing respiratory substrate, P_i and Mg^{2+}). Under these conditions the mitochondria are tightly coupled so addition of ADP increases O_2-consumption until all exogenous ADP is phosphorylated and the O_2-consumption returns to original level (A). Addition of DNP causes a more marked stimulation of O_2 consumption (B), In contrast oligomycin (OL) does not inhibit the respiration rate but blocks the ADP-stimulation of O_2-consumption (C), however, DNP will still stimulate O_2 consumption in the presence of oligomycin (C). Addition of oligomycin also prevents the ADP stimulation of O_2-uptake (D).

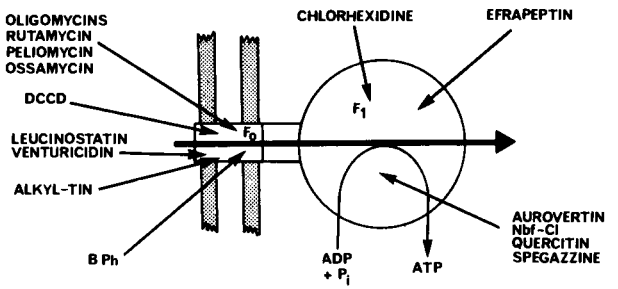


Figure 4.5. Inhibitors of bacterial and mitochondrial membrane-bound Mg^{2+}-ATPase.

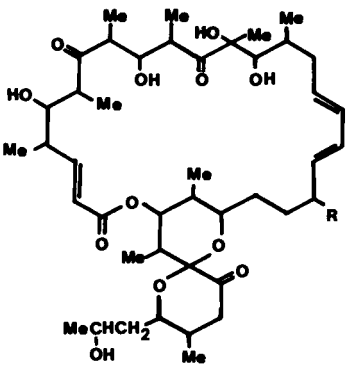
the F_0 or BF_0 complex (*Figure 4.5*). It will be shown later that each of the inhibitors described in this review has a specific binding site in the ATPase complex. The use of synchronous cultures of the fission yeast *Schizosaccharomyces pombe* has allowed the temporal resolution of the inhibitor-sensitivity of the mitochondrial Mg^{2+}-ATPase complex [99—101]. The inhibitors of the

F_0 component can be divided into two groups; site I includes oligomycin, venturicidin and alkyl-tins while site II includes *N,N'*-dicyclohexylcarbodiimide (DCCD) and leucinostatin. Similarly, agents capable of inhibiting the F_1 region can be separated into five other distinct sites *i.e.*, efrapeptin (III), Nbf-Cl (IV), aurovertin (V), quercitin (VII) and spegazzine (VIII).

INHIBITORS WHICH INTERACT WITH THE F_0-REGION

OLIGOMYCINS AND RUTAMYCIN

Oligomycin is a macrolide antibiotic mixture produced by a species of Actinomycete similar to *Streptomyces diastatochromogenes* [102—104] and was shown later to be produced by *Aspergillus* sp. and *Glomerella* sp. The producing strains give a complex of several closely-related compounds designated A, B and C [104], the proportion of each in the mixture depending upon cultural conditions [105]. Oligomycins B and C were stable but oligomycin A slowly lost activity [106]. The structure of oligomycin B (1) has been determined [107—111]. Oligomycin A differed from oligomycin B only in the replacement of carbonyl oxygen in the bottom pyran ring by two hydrogen atoms [111]. Oligomycin C was similar to oligomycin A excepting replacement of the tertiary hydroxyl group at the top of the macrolide ring by a hydrogen atom [111]. Rutamycin, originally Lilly A272, was isolated from an actinomycete, probably a strain of *Streptomyces rutgerensis* [112]. Rutamycin (2) is a homologue of oligomycin B, possessing a methyl side-chain in the place of an



(1) Oligomycin B, R = Et
(2) Rutamycin, R = Me

ethyl group in the large lactone ring [111,113]. Rutamycin so resembles the oligomycins in many of its chemical, spectrophotometric and biological properties that it has been termed oligomycin D.

Oligomycin was shown to have little effect against Gram-positive or Gram-negative bacteria and many yeasts but was strongly inhibitory to a limited number of fungal species (hence the name *oligo*mycin) [102,103]. The antibiotic has been suggested as a useful topical antifungal agent, particularly against the human pathogen *Blastomyces dermatitidis*. Like oligomycin, rutamycin has no antibacterial activity and only a limited antifungal spectrum [112]. The mammalian toxicity of oligomycin is high (in mice, Ld_{100} value for intravenous route, 2.5 mg/kg body wt., and Ld_{50} value for intraperitoneal route 1.5 mg/kg body wt.) [102].

Oligomycins inhibit the oxidation of a variety of substrates by intact mitochondria and ion transport in cells where active uptake is ATP-dependent, *i.e.*, in fungi and certain yeasts. The relative effectiveness of oligomycins and rutamycin has been determined [106,114]. Their inhibitory abilities varied, depending upon the substrate used, but in general oligomycin A was the most effective and oligomycin C was the least active [106,114]. This correlates with the reported effectiveness of oligomycins in the inhibition of fungal growth (*i.e.*, A > B > C) [104]. Rutamycin has been shown to be as potent an inhibitor of mitochondrial ATPase as are oligomycins A and B [106,111,113].

Rutamycin and oligomycin do not bind to or inhibit isolated F_1, but both are inhibitors of the membrane-bound complex [30]. Inhibition of mitochondrial respiration by oligomycin is completely reversed by uncouplers, *e.g.* DNP (*Figure 4.4*, trace D), which suggests that the antibiotic does not act upon the electron transport chain itself but acts upon some phase of energy-transfer associated with the terminal step of the electron transfer sequence, *i.e.* the incorporation of inorganic phosphate P_i into ATP [115]. Oligomycin blocked the ability of mitochondria or submitochondrial vesicles to synthesize ATP, but unlike uncouplers, did not cause dissipation of the high energy state of the mitochondrial membrane. The antibiotic reduced oxygen consumption of both intact cells (*Figure 4.4*) and certain submitochondrial preparations but unlike those agents which blocked the respiratory chain, the effect of oligomycin was only evident in tightly coupled mitochondria. In well-coupled systems oligomycin concentrations sufficient to inhibit only partially oxygen consumption proportionally inhibited phosphate incorporation; that is, oligomycin has little effect on the P/O ratio [114]. Oligomycin had little effect on respiration in submitochondrial preparations but phosphorylation was completely blocked [116—118].

The ability of oligomycin complex to prevent ATP hydrolysis induced by a wide range of uncouplers has received extensive study [113—120]. Oligomycin A caused an 80—100% inhibition of the uncoupler-induced ATPase activity. Rutamycin and oligomycin A and B showed approximately equal activity, but oligomycin C was somewhat less active. The antibiotic also inhibited uncoupler-induced ATPase activity in submitochondrial preparations [116]. The effect of oligomycins and rutamycin upon another event associated with oxidative phosphorylation, namely, the exchange reaction involving ADP, ATP and P_i, has also been studied. The exchange of ^{32}P into, and the loss of ^{18}O from ^{18}O-labelled phosphate of, ATP in intact mitochondria was rapidly inhibited by low concentrations of oligomycin or rutamycin [121—123]. The uptake of ions and metabolites by bacteria or mitochondria is often against a concentration gradient and thus requires the expenditure of metabolic energy. The uptake of P_i, Mn^{2+} and Mg^{2+} by beef heart or rat liver mitochondria has been shown to be insensitive to oligomycin but readily inhibited by uncouplers [124,125], *i.e.* uptake was not ATP-dependent. In the presence of inorganic phosphate, isolated mitochondria begin to swell and since the addition of ATP can reverse this swelling, it has been suggested that the swelling phenomenon is related to electron transport and subsequent energy transformation. Oligomycin and rutamycin prevent phosphate-induced mitochondrial swelling in the presence of β-hydroxybutyrate or glutamate [126,127]. On the addition of uncoupler in the presence of either substrate, this can be reversed. However, in the presence of glutamate uncoupler, reversal only occurred if ADP was present, ADP apparently serving as the phosphate acceptor for obligatory phosphorylation [128]. It was suggested that, as oligomycin inhibits both phenomena related to ATP synthesis and the hydrolysis of ATP in mitochondria, ATP utilization in mitochondria involves a reversal of the ATP generating mechanism. It has been demonstrated that potassium chloride enhances the inhibitory effect of oligomycin in mitochondria [129].

As respiration may proceed in oligomycin-treated submitochondrial preparations in the presence of uncoupler, it was suggested that the effect of oligomycin on respiration was indirect and due to inhibition of those stages of oxidative phosphorylation normally coupled to respiration in intact cells. Submitochondrial preparations may be made in such a way that they are devoid of F_1. Such preparations were no longer capable of ATP synthesis or P_i exchange and were not bound or sensitive to oligomycin. Complete oligomycin sensitivity can be restored by recombination with suitable F_1 preparations [30,130,131]. Further analyses suggested that the presence of a small basic protein (mol. wt. 18 000), the oligomycin sensitivity-conferring

protein (OSCP) and a component of the link between F_1 and the F_0 region of the mitochondrial membrane, was necessary for oligomycin sensitivity [132,133]. Mitochondrial membranes, like all other membranes, are inherently impermeable to protons [134—138], although permeability is increased by the addition of uncouplers or during ATP hydrolysis and the transport of nutrients which require a proton symport or antiport (*Figure 4.1*). The translocation of protons across the energy-transducing membrane through the F_0 complex [137,138] is concomitant with ATPase or ATP synthetase activity (*Figure 4.1*). Vesicles made from mitochondrial membranes stripped of the F_1 complex either chemically, physically or by genetic deletion exhibit markedly increased proton conductance [139—141] and decreased efficiency of respiration-linked energy-dependent reactions. When such preparations were treated with oligomycin, rutamycin (or DCCD) or by addition of the OSCP/F_1/F_6 complex, the proton conductance fell and the energy transducing properties were substantially repaired [142—148]. It is suggested that F_1 has both a structural and a catalytic role in the membrane ATPase and that in the absence of F_1 the structural role can apparently be simulated by the inhibitor. Oligomycin, by binding to the F_0 complex, blocks the proton-translocating channel of the F_0 thereby preventing the dissipation of the PMF in F_1-less particles. By binding to the intact mitochondrial membrane-bound ATPase, the antibiotic deprives the ATP synthetase of the proton flow necessary for ATP synthesis. The oligomycin binding site has been purified from yeast mitochondria and identified as a low molecular weight proteolipid (mol. wt. 8000) which was absent from oligomycin-resistant mutants [149] and which may also serve as the binding site for DCCD [150]. There is some evidence to suggest that sulphydryl groups are involved in the antibiotic-enzyme interaction [151]. Extraction of the membrane bound ATPase of oligomycin-resistant mutants of *Aspergillus nidulans* with Triton-X100 showed that the enzyme was eighty times more resistant to the antibiotic than was the wild type enzyme. It was suggested that a small lipophilic protein, coded by mitochondrial genes, determined oligomycin-resistance in this species and this proteolipid was synthesised on mitochondrial ribosomes before incorporation into the membrane sector of the ATPase [152—154]. An F_0 proteolipid subunit of oligomycin-sensitive Mg^{2+}-ATPase from *Saccharomyces cerevisiae* was found to be normally present in a high molecular weight form [155], while resistant mutants were found to contain a proteolipid of lower molecular weight. Mutants of the yeast *Kluyveromyces lactis* resistant to oligomycin have been isolated [156,157]; two nuclear and three cytoplasmic mutations conferring oligomycin-resistance were described [156]. Nuclear mutations conferring oligomycin resistance in *Saccharomyces*

cerevisiae have been recorded but in this case the mitochondrial ATPase of the resistant mutant appeared to be as sensitive as the wild type [157], leading to the suggestion that in this case the mutation affected the permeability properties of the cell envelope, preventing access of the drug to the enzyme. The mutation of a nuclear gene has also been shown to confer oligomycin resistance in *Neurospora crassa* [158] but this mutation did not decrease the sensitivity of the enzyme to F_1 inhibitors.

Rutamycin, like oligomycin, does not bind to purified F_1 [30,130] but binds to mitochondrial vesicles devoid of F_1 when containing F_0 and OSCP [106]. Subsequent addition of F_1 and phospholipids results in a complex incapable of ATP hydrolysis, suggesting that rutamycin, like oligomycin, binds to an F_0 component. A rutamycin binding factor (F_c) has been isolated from beef heart mitochondria [159]. This protein does not bind DCCD suggesting that rutamycin has a unique binding site in the F_0-complex.

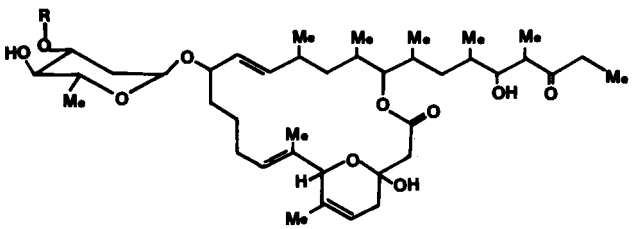
Neither purified nor membrane-bound bacterial ATPase is inhibited by oligomycin [13]. The BF_1 component of the baterial ATPase, like that of mitochondria, is attached to the BF_0 region of the membrane by a stalk (*Figure 4.3*). It appears that the δ-subunit of the BF_1 of *E. coli* Mg^{2+}-ATPase is functionally analogous to the OSCP of mitochondria and, as this subunit is incapable of binding oligomycin or rutamycin, the bacterial system is by its very nature insensitive.

OSSAMYCIN AND PELIOMYCIN

These antibiotics produced by *Streptomyces luteogriseus* and *hygroscopicus* respectively [160—162] possess little antibacterial action, some activity against yeasts and moulds and extensive cytotoxicity. Ossamycin showed acute mammalian toxicity (LD_{50} values in mice, intravenous route, 1.8 mg/kg, subcutaneous route, 2.3 mg/kg and intraperitoneal route, 1.8 mg/kg [160]). Peliomycin is believed to be chemically similar to oligomycin B [111] while ossamycin may be similar to venturicidin [111]. Both were potent inhibitors of membrane-bound ATPase but had no effect on purified F_1 (163). The activity of ossamycin and peliomycin has been compared with rutamycin. On a weight to weight basis, peliomycin was equivalent to rutamycin in its inhibitory activity, whereas ossamycin is much less active [163].

VENTURICIDINS

Venturicidins are antifungal antibiotics produced by a strain of *Streptomyces aureofaciens* and named for their toxicity towards the fungus *Venturia*



(3) Venturicidin A, R = $CO.NH_2$
(4) Venturicidin B, R = H

inaequalis [164]. Venturicidins A (3) and B (4) possess a lactone ring similar to that of oligomycin B but smaller (a twenty atom ring compared with a twentysix atom ring for oligomycin) [111, 165—167]. The dideoxycarbamide sugar is not essential for antibiotic activity. Venturicidin B which has no carbamido group was as potent as Venturicidin A [111]. Venturicidin X, the aglycone was found to be three times more active than was the parent compound [111]. The antibiotic was shown to be toxic to a wide range of fungi while exhibiting low oral, intravenous or intraperitoneal toxicity [164].

Venturicidins share many common properties with rutamycin but differ in that even at high concentrations they fail to completely inhibit coupled phosphorylation in mitochondria [163]. At lower concentrations, they appeared less effective than rutamycin in inhibiting uncoupler-induced ATP hydrolysis. Venturicidins have been shown to be more effective in inhibiting the Mg^{2+}-ATPase of yeast mitochondria than oligomycin [168]. Yeast mutants insensitive to oligomycin remained sensitive to venturicidin, suggesting that venturicidin acts at a site close to, but distinct from, those of oligomycin and rutamycin [168]. Extraction of an oligomycin-sensitive F_1/F_0 complex from beef heart mitochondria showed that the sites of action of DCCD, oligomycin and venturicidin were functionally separated [169]. Triton-treatment of this complex modified its response to inhibitors. In triton-treated complexes, venturicidin was shown to stimulate rather than inhibit the ATPase [169]. Ossamycin may be similar to venturicidin A as it also contains a carbamino sugar and mimics venturicidin in some of its properties [111].

LEUCINOSTATIN AND A20668

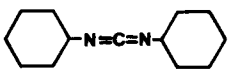
This polypeptide antibiotic, isolated from *Penicillium lilacinum*, was so named as leucine was the predominant hydrolysis product [170]. A closely related polypeptide antibiotic A20668, molecular weight 1570, also gave pre-

dominantly leucine on hydrolysis [111]. These antibiotics were inactive against Gram-negative bacteria, moderately active against Gram-positive bacteria, and very active against a wide range of pathogenic and non-pathogenic moulds and yeasts [170]. The antibiotics are extremely toxic to tissue culture cells [170] and to animals (LD_{50} value in mice, intraperitoneal route, 1.6 mg/kg) [170,171].

A20668 has been shown to inhibit uncoupler-induced ATP-hydrolysis but, as an inhibitor of Mg^{2+}-ATPase in submitochondrial preparations, it was less effective than rutamycin [172]. A20668 has been shown to have an inhibitory action similar to that of oligomycin, but high concentrations of A20668 appeared to uncouple oxidative phosphorylation *i.e.*, A20668 acts both as an F_0-binding inhibitor and at higher levels as a classical uncoupler. It has been proposed that leucinostatin and A20668 bind at a site distinct from oligomycin, venturicidin or ossamycin [111]. As tissue culture cells resistant to rutamycin were also resistant to leucinostatin, it has been proposed that the leucinostatin binding site is close to that of rutamycin on the inner mitochondrial membrane [173]. Studies with liposomes indicate that the initial interaction of leucinostatin with the enzyme requires the participation of membrane phospholipid [174]. Leucinostatin inhibited photosynthetic and oxidative phosphorylation and related reactions, that is, dark and light ATP-P_i exchange reactions, and the Mg^{2+}-ATPase in chromatophores of the non-sulphur photosynthetic bacterium *Rhodospirillum rubrum*. Isolated chromatophores from this organism were also sensitive to oligomycin and auvovertin [175—177].

N,N'-DICYCLOHEXYLCARBODI-IMIDE (DCCD)

Carbodi-imides have been reported to be effective herbicides [178—180] and to possess insecticidal, nematocidal and fungicidal properties [178,179]. DCCD (5) has been shown to be a potent inhibitor of mitochondrial Mg^{2+}-



(5) DCCD

ATPase [181]. At first sight the mechanism of action of DCCD appears to be similar to that of oligomycins and rutamycin, being an inhibitor of membrane bound Mg^{2+}-ATPase, while having no effect on the soluble enzyme (F_1). DCCD, like oligomycin, at concentrations that inhibit oxidative phosphory-

lation suppressed proton conductance in submitochondrial [145,146,182] and bacterial [147,148] vesicles devoid of soluble enzyme. However, there are several indications that oligomycin and DCCD attack different components in the proteolipid membrane region of the enzyme complex. Bacteria, which because they do not contain OSCP are oligomycin-resistant, have been shown to be DCCD-sensitive [76]. An isolated Mg^{2+}-ATPase from beef heart mitochondria has been shown to be oligomycin-rutamycin sensitive, but DCCD-resistant [29]. Treatment of mitochondria with the detergent Triton X-100 was shown to abolish specifically the sensitivity of the enzyme complex to DCCD but oligomycin sensitivity was unchanged [169]. DCCD has been shown to interact with mitochondrial preparations made in such a way that they contain no OSCP [183] while isolated OSCP failed to bind DCCD [159]. Detachment of the F_1 portion of the enzyme from yeast submitochondrial vesicles increases the affinity of the preparation for oligomycin whereas the affinity towards DCCD remained unchanged [184]. These data suggest that DCCD acts at a site distinct from oligomycin and that this site is located closer to F_0 than OSCP [185,186].

Although membrane-bound Mg^{2+}-ATPase from mitochondria and bacteria are readily inhibited by DCCD, purified F_1 preparations were insensitive [142,187—189] *i.e.* the DCCD-binding component must be located in the membrane. DCCD has been demonstrated to bind covalently to a proteolipid component of the F_0 complex of mitochondria [142,187,190—193] at a site distinct from oligomycin, rutamycin and venturicidin [100,169]. This component, designated the DCCD sensitivity conferring complex (DSCP) has been isolated from mitochondria of yeasts, *Neurospora* and mammalian cells [191,194,195] and bacteria [196]. The properties of mitochondrial DSCP have been investigated using a DCCD derivative and it has been shown that DSCP interacts in some way with OSCP [197].

Carbodi-imides are commonly used as condensing reagents in the preparation of synthetic peptides [198—200]. The chemically reactive carbodi-imide moiety, believed to serve as the linking agent in peptide syntheses, also appeared to be essential for Mg^{2+}-ATPase inhibition [191]. Water-soluble carbodi-imides from stable links with several functional groups on proteins, (*e.g.*,—COOH,—Tyr—OH,—SH,—Ser—OH) [201—203]. The potency of various carbodi-imides as inhibitors of Mg^{2+}-ATPase from *S. aureus* has been shown to related directly to their lipophilicity [204]. Water soluble carbodi-imides react with proteins in aqueous solution more effectively than lipophilic carbodi-imides, *e.g.*, DCCD. However, the water-soluble carbodi-imides require concentrations 100 to 1000 times greater than DCCD to inhibit the Mg^{2+}-ATPase, reflecting the membrane location of DSCP; hydrophobic

carbodi-imides by partitioning more easily into the lipid phases being better able to reach local concentrations sufficiently high to modify the functioning of F_0.

The membranes of *E. coli* possess a prominent Mg^{2+}-ATPase which was inhibited by DCCD [188,205,206] as were other reactions related to energy-transduction, *e.g.* oxidative phosphorylation and ATP-driven metabolite uptake by anaerobic or cyanide-treated cells [207—210]. DCCD-inhibition of the membrane bound Mg^{2+}-ATPase of *E. coli* was dependent upon the cation composition of the medium [211], a rise in cation concentration increasing the inhibitory effect of DCCD. It is possible to isolate from the membranes of *E. coli* a BF_1/BF_0 complex which retains DCCD-sensitivity [5,196,212]. The partially-purified DCCD-sensitive complex consisted of twelve polypeptides. In addition to the α to ε subunits of the BF_1, two other peptides ξ and η (mol. wt. 29 000 and 9000 respectively) were identified, one of which bound DCCD [213]. Treatment with [^{14}C]DCCD has shown that η corresponded to DSCP for *E. coli* [196,213—215]. The Mg^{2+}-ATPase of *Streptococcus faecalis* has been purified and been shown to exhibit no ADP—ATP exchange activity or apparent phospho-enzyme derivative [73,76,204]. DCCD inhibited this enzyme and a number of energy-dependent transport processes in the intact organism [142,216]. Although the solubilized enzyme was unaffected by DCCD the membrane-bound enzyme was sensitive. Active ATPase was isolated from DCCD-inhibited membrane preparations, but active ATPase added to DCCD-treated membranes was found to lose activity [142,216]. It has been demonstrated that the inhibitor binds to a component of the BF_0 of *S. faecalis*, the carbodi-imide sensitivity factor (CSF) [217], possibly to an undissociated carboxyl group of the CSF [218]. Like uncouplers, DCCD has been shown to dissipate the established pH gradient present across the cell membrane of *S. faecalis* [73,204]. This suggests that ATP hydrolysis may be linked to the establishment of the proton gradient, consistent with the 'chemiosmotic' hypothesis of energy transduction. DCCD has also been shown to inhibit the membrane-bound Mg^{2+}-ATPase of *Bacillus subtilis* [67] and the obligate anaerobe *Clostridium pasteurianum* [219] but had no effect on the purified soluble enzyme from either organism. Addition of DCCD to anaerobic suspensions of *E. coli* caused a decrease in proton translocation coupled to fumarate reduction by endogenous substrate and the half-time of proton entry after translocation [220], *i.e.*, binding of DCCD to BF_0 appeared to block the proton conducting channel, thereby depriving the enzyme of its driving force. DCCD also inhibited fumarate reduction in cell-free extracts [220].

DCCD-resistant mutants of *S. faecalis* and *E. coli* have been isolated

[214,218] and shown to grow normally showing unimpaired capacity for energy transduction and ability to carry out ATP-dependent membrane functions. However, the membrane-bound Mg^{2+}-ATPase was more than one hundred times less sensitive to the inhibitor than was the wild-type enzyme. The assembly of hybrids from wild-type and DCCD-resistant mutants showed that DCCD-sensitivity was conferred by a component of the BF_0. Since the membranes of these and other mutants contained a DSCP, it was suggested that DCCD-resistance was the result of changes in the proteolipid structure of BF_0, in such a way that those functional groups conferring DCCD-sensitivity were no longer accessible to the inhibitor. Several UncB (uncoupled) mutants of *E. coli* were also shown to be resistant to DCCD. The inhibitor restored defective respiration-linked reactions of two of the mutants, although the third was unaffected [210,221,222]. In these mutants DCCD-sensitivity depended on the presence of an additional DCCD-binding protein, other than DSCP, apparently necessary for the binding of BF_1 to the coupling membrane [223]. Other DCCD-resistant *E. coli* mutants, closely related to UncB genes, have been shown to possess an inhibitor-insensitive Mg^{2+}-ATPase [224]. The mutation appeared to affect the membrane-integrated portion of the enzyme *i.e.*, BF_0. An UncB mutant with a DCCD-resistant Mg^{2+}-ATPase has been shown to be only capable of membrane energization at the expense of respiration [225]. The BF_0 complex of this mutant was found to lack a 50 000 molecular weight polypeptide present in the wild-type [225]. It was concluded that in this case DCCD-resistance was not a lesion in the DSCP but rather reflected changes in the way the BF_1 component was bound to BF_0 and this modification prevented access of DCCD to its binding site.

ORGANOTIN COMPOUNDS

Organic compounds of tin have found use as fungicides [226,227], insecticides [227—230] and industrial biocides [227,231—234]. Trialkyltin compounds, such as tributyltin, triethyltin or triphenyltin, although bacteriostatic to a wide range of Gram-positive bacteria [227,235] exhibit little activity against Gram-negative bacteria [227,236]. Organotin compounds show acute mammalian toxicity [227,236—239]. These compounds have never found use as agricultural insecticides or fungicides due to their phytotoxicity [227,240,241], being equally inhibitory to the photosynthetic generation of ATP [241] or oxidative phosphorylation in insect [229], fungi [184] and mammalian [242,245] mitochondria. Fungicidal activity of alkyltins has been shown to be dependent upon the extent of alkylation being maximal in tri-alkylated compounds, triethyltin being at least one hundred times more fungicidal

than diethyltin [226]. At neutral pH triethyltin was a specific and potent inhibitor of mitochondrial energy conversion in yeasts, preventing growth of the organism on oxidizable substrates but under the same conditions diethyltin had little effect [242].

On treatment of mitochondria with trialkyltins, two distinct phenomena were observed; general effects in which the compound appeared to act as an uncoupler and specific inhibitory activity of coupled mitochondrial respiration and phosphorylation. In the latter trialkyltins appeared to act in a way similar to oligomycin in that they not only inhibited oxidative phosphorylation, but also partial reactions such as P_i-ATP exchange and ATPase activity [183,185,241,243—245] and caused mitochondrial swelling [246]. Alkyltin compounds have been shown to inhibit membrane bound Mg^{2+}-ATPase but to have no effect on isolated soluble enzyme [130,247]. Like DCCD and oligomycin, trialkyltins decreased the proton permeability of F_1-depleted submitochondrial preparations [183]. These data suggest that organotin compounds bind to the F_0 region of the membrane. The binding site, however, appears to be distinct from that of DCCD or oligomycin [185]. Rat liver mitochondria were found to possess at least two sets of organotin binding sites, the site with the higher affinity for the inhibitor appearing to be the site involved in oxidative phosphorylation [246]. Two distinct binding sites have also been postulated for yeast Mg^{2+}-ATPase, where extraction of an oligomycin-sensitive F_1/F_0 complex with detergent revealed the presence of six triethyltin binding sites/molecule of enzyme complex. Removal of OSCP and F_1 from the complex did not reduce binding and it was proposed that the triethyltin binds to three proteolipid components of the yeast F_0 [247], possibly by the chelation of histidine groups [242,243]. Examination of *Saccharomyces cerevisiae* mutants resistant to organotin compounds also suggested that the compounds bind to a unique component of the F_0 region [169,248—250]. Mitochondria from yeast strains resistant to trialkyltin showed cross-resistance to bongkrekate, a known inhibitor of ADP-translocase, suggesting the possibility that the membrane bound ATP synthetase complex and the ADP translocase have a common protein subunit [249].

BATHOPHENANTHROLINE (4,7-DIPHENYL-1,10-PHENANTHROLINE)

Bathophenanthroline (BPh) is a lipophilic compound able to inhibit both electron transport and the membrane bound Mg^{2+}-ATPase of both bacteria and mitochondria. BPh did not act upon the soluble enzyme (BF_1) or the membrane bound enzyme of UncA *E. coli* mutants [251—253]. It has been

suggested that BPh may chelate membrane iron and the resultant inhibition of electron transport halts ATP synthesis [251,254]. Mutation in either the UncA or UncB gene of *E. coli* caused a reduction in the degree of inhibition of membrane-bound Mg^{2+}-ATPase activity without affecting the NADH oxidase activity suggesting a common binding site with BPh [255,256]. BPh inhibition of membrane bound mitochondrial and bacterial ATPase may be reversed by uncouplers [255—257]. It has been suggested that in mitochondria BPh interrupts energy transfer somewhere between the electron transport system and the site of action of oligomycin [257,258]. The observation that BPh inhibition of beef heart mitochondria is antagonized by ATP suggests that the inhibitor may also modify the region of the F_1 involved in regulation of the enzyme [259].

INHIBITORS WHICH INTERACT WITH THE SOLUBLE ENZYME

AUROVERTIN

This antibiotic produced by *Calcarisporium arbuscula* was named for its yellow colour and in the mistaken belief that it was produced by a species of *Verticillium* [260]. Aurovertin has been purified as a yellow crystalline compound with a molecular formula of $C_{25}H_{32}O_9$ [261]. The antibiotic possesses acute mammalian toxicity (intravenous LD_{50} values, mice 1.6 mg/kg, rabbit 0.5 mg/kg) but showed little effect against a range of Gram-negative and Gram-positive bacteria or pathogenic fungi, although some anti-protozoal activity was noted [260].

Aurovertin has been shown to be a more potent inhibitor of mitochondrial Mg^{2+}-ATPase than oligomycin or DCCD and like oligomycin its effects were reversed by the addition of uncouplers but did not itself act as an uncoupler of intact mitochondria [114,261]. However, aurovertin differs in its mode of action from oligomycin and DCCD. The latter inhibitors showed equal inhibition of ATP-utilizing and ATP-generating systems but, although aurovertin was more effective than oligomycin and DCCD in halting ATP synthesis, inhibition of ATP-utilizing systems was much less [261,262]. Aurovertin was apparently more effective in blocking the forward reaction of ATP synthesis in oxidative phosphorylation than the reverse energy transfer reaction [114,263—266]. Unlike oligomycin and DCCD, aurovertin inhibits soluble Mg^{2+}-ATPase of mitochondria [267], and bacteria [268]. The binding of aurovertin to F_1 creates a flurophore [269—271] allowing the determination of the stoichiometry of aurovertin binding [111,272—274]. It was

originally suggested that F_1 bound one molecule of aurovertin/molecule F_1 but later it was shown that, in the presence of ATP, F_1 contained two sites for aurovertin binding, of equal affinity for the inhibitor, but in the absence of ATP only one binding site was present [111,263,270,273]. The suggestion that aurovertin binds to the β-subunit of F_1[183] has been confirmed in beef heart mitochondria where it was shown that aurovertin bound to isolated β-subunit by a conventional three-point binding reaction with one aurovertin binding site per subunit [274]. Aurovertin-resistant mutants of *Saccharomyces cerevisiae* were shown to carry a mutation in the structural gene for one of the three larger protein subunits of the F_1 complex (*i.e.*, α, β, or γ) [275]. Isolated β-subunits from purified yeast F_1 were incapable of ATP hydrolysis but still able to bind aurovertin. β-subunits from aurovertin-resistant mutants, however, were unable to bind the inhibitor [276].

EFRAPEPTIN (A 23871)

This polypeptide antibiotic is a potent inhibitor of mitochondrial Mg^{2+}-ATPase. It appears to bind to the locus at which nucleotides bind during ATP syntheses and hydrolysis [111,277]. Binding of aurovertin and efrapeptin at this site was not mutually exclusive [111]. Efrapeptin inhibited photosynthetic and oxidative phosphorylation in chromatophores of the photosynthetic bacterium *Rhodospirilium rubrum* and was capable of inhibiting the soluble Ca^{2+}-activated ATPase of the chromatophore [278]. The antibiotic was not competitive with ATP suggesting that efrapeptin was an inhibitor of energy transfer acting upon the soluble ATPase. It has been demonstrated that efrapeptin binds to the CF_1 of spinach chloroplasts [279].

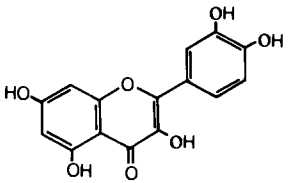
4-CHLORO-7-NITROBENZOFURAZAN (Nbf-Cl)

Nbf-Cl, also known as 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole, was synthesized as an intermediate in a synthetic route to 4-aminobenzofurazans [280]. This compound bound covalently to both the soluble and membrane-bound bacterial or mitochondrial ATPase [60,281—284] inhibiting ATP hydrolysis and ADP respiratory control by energy-transducing membranes. The action of Nbf-Cl may be reversed by treatment with the sulphydryl reagent, dithiothreitol, apparently by removal of the Nbf group from the enzyme [60,281]. Nbf-Cl has been shown to react with the phenolic oxygen of tyrosine and the sulphydryl group of cysteine [281,282,285,286]. Nbf-Cl has been shown to bind to the β-subunit of mitochondrial F_1 [60] and, as the inhibitory action was reversed by addition of nonpenetrating sulphydryl reagents, *e.g.*

glutathione or dithiothreitol, it has been suggested that the affected tyrosine residues were surface-located [285]. Modification of a single tyrosine residue of beef heart mitochondria by Nbf-Cl was sufficient to modify the conformational behaviour of the enzyme [281,283,287,288] and it has been suggested that this particular tyrosine residue participates in the rate-determining donation of protons to ATP. Nbf-Cl also bound to, and modified, the β subunit of the BF_1 complex of *Paracoccus denitrificans* [285], *E. coli* [37,60] and *Alcalcigenes faecalis* [268]. However, although some Nbf-Cl was bound by the β-subunit of *E. coli*, it was later shown that the inhibitor was predominantly bound by the α-subunit [284]. The α-subunit of *E. coli* appeared to possess properties that in other Mg^{2+}-ATPase were associated with the β subunit [284]. However this distinction may be of little importance, since the subunit nomenclature is based exclusively on their mobility on SDS polyacrylamide gels and has no functional or structural significance.

QUERCITIN

This compound, widely distributed in plants (particularly in tree barks), was first extracted from *Rhododendron cinnabarium* [289] and shown to be 3,3′,4′,5,7-pentahydroxyflavone (6) [290]. It is an active inhibitor of both



(6) Quercitin

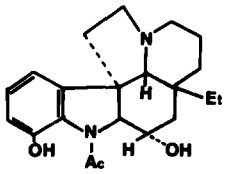
soluble and membrane bound mitochondrial Mg^{2+}-ATPase and it has been proposed that quercitin acts in a way similar to that of the Mg^{2+}-ATPase inhibitor peptide (I) [40,291] which is believed to regulate ATP hydrolysis. Although quercitin is toxic if taken internally, metallic complexes of the compound have been proposed as topical antimicrobial agents [292]. Quercitin also possess viricidal properties [293].

Quercitin presumably modifies a component of F_1 by interaction with protein sulphydryl or amino groups [294]. A comparison of various flavones indicated that the 3′-, and perhaps the 3-, hydroxyl groups were important in the mechanism of inhibitory activity [291] and antiviral action [293]. Unlike mitochondrial inhibitor protein, quercitin also inhibited the ATP-dependent

reduction of NAD^+ by succinate in reconstituted submitochondrial particles [291]. Quercitin has also been shown to modifiy the Na^+/K^+-transport ATPase [295,296]. The compound also inhibited the membrane bound Mg^{2+}-ATPase of chloroplast membranes, by binding to the β subunit, blocking the site of ATP binding and inhibiting photophosphorylation [297,298]. Quercitin has also been demonstrated to bind to the β-subunit of purified BF_1 from *E. coli* [299,300].

SPEGAZZINE

Spegazzinine, a dihydroindole alkaloid, was first isolated from the bark of *Aspidosperma chakensis* [300] and its structure determined [301,302] as 3-hydroxy-17-dimethylaspidospermine (7). At low concentrations spegazzinine



(7) Spegazzinine

inhibited oxidative phorphorylation [303] but was less effective in halting ATP-dependent reactions, causing only partial inhibition of membrane-bound and soluble mitochondrial ATPase [303]. It has been suggested that spegazzinine acts at a site on the mitochondrial F_1 at or near the site of aurovertin action [303].

DIO-9

This antibiotic has received little attention and is of unknown structure. It is active against certain pathogenic and non-pathogenic bacterial and fungi [304]. In the absence of P_i,DIO-9 acted as an uncoupler but in the presence of P_i was a potent inhibitor of rat liver membrane bound Mg^{2+}-ATPase [304—306]. DIO-9 inhibition of rat liver mitochondria, in the presence of P_i, was reversed by addition of DNP [304,305]. The inhibitor also caused mitochondrial swelling [305] and halted P_i-ATP exchange in rat liver mitochondria, *i.e.*, DIO-9 acts like a true Mg^{2+}-ATPase inhibitor [303,304]. DIO-9has been shown to inhibit both membrane bound and soluble Mg^{2+}-ATPase of yeast mitochondria, *i.e.* DIO-9 must act on the F_1 region of the

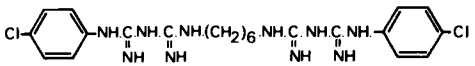
yeast enzyme [53,307]. DIO-9 also inhibited the soluble and membrane bound Mg^{2+}-ATPase of *S. faecalis*, halting net K^+-uptake, without affecting membrane bound NADH dehydrogenase [308].

OCTYLGUANIDINE

This compound inhibited the ATPase activity of membrane bound and soluble mammalian mitochondrial Mg^{2+}-ATPase [309,310] and may act in a similar way to that of the natural ATPase inhibitor protein (I) [311].

CHLORHEXIDINE

This compound is used widely as an antiseptic. It contains two strongly basic biguanide groups (8). It is much less surface-active than cationic surfactants



(8) Chlorhexidine

and is often formulated as the gluconate which possess better stability in aqueous systems. The compund is well tolerated by mammalian tissues and has been shown not to be toxic when applied to skin or mucosal membranes [312]. Chlorhexidine has found use in preoperative skin disinfection, wound irrigation, in obstetrics, urology and treatment of burns. At low concentrations the antiseptic was lethal to both Gram-negative and Gram-positive bacteria, although showing more activity against the latter [312—314]. The agent had little antifungal, sporicidal or antimycobacterial activity, was inactive against *Pseudomonas* sp., and was neutralized by organic matter or Lubrol W [312,315].

At low concentrations chlorhexidine, like other cationic antiseptics, induced the leakage of low molecular weight cytoplasmic components from *E. coli, Staph. aureus* [315—317] and *Micrococcus lysodeikticus* [318,319] but at higher antiseptic concentrations leakage was prevented, presumably by precipitation of the cytoplasm.

At minimum inhibitory concentrations (10^{-5}M) chlorhexidine was shown to be a direct inhibitor of bacterial Mg^{2+}-ATPase, inhibiting net K^+—Na^+ exchange in *S. faecalis* [308]. Both membrane bound and solubilized Mg^{2+}-ATPase from aerobic (*S. faecalis*) [308] and anaerobic (*Clostridium perfringens*) [320] bacteria were inhibited. Inhibition of the solubilized enzyme

suggested that chlorhexidine acted directly upon BF_1 and that the associated inhibition of K^+-transport provided additional evidence that Mg^{2+}-ATPase and ion translocation were coupled [308].

CONCLUSIONS

Selective toxicity, that is, the ability to damage the desired target without affecting the host, is the basis of all successful therapeutic agents. The intrinsic similarity in the physiological, bioenergetic and biochemical properties of energy transducing membranes of all living cells makes it particularly difficult to find agents acting upon membrane enzymes possessing any degree of selective toxicity. Few of the many inhibitors of membrane-bound Mg^{2+}-ATPase inhibitors described have ever been used therapeutically other than as antiseptics or for topical application. This is not to say that the extensive study of inhibitors of Mg^{2+}-ATPase has been wasted. In fact, these compounds have proven to be invaluable tools in the study of membrane bioenergetics. Their use has led to an understanding of the function of Mg^{2+}-ATPase of all living systems, particularly in the differentiation of the membrane-bound proton translocating region of the enzyme (F_0) from the site(s) of ATP hydrolysis and syntheses.

REFERENCES

1. A.E. Senior, Biochim. Biophys. Acta, 301 (1973) 249.
2. R.D. Simoni and P.W. Postma, Annu. Rev. Biochem., 44 (1975) 523.
3. P. Postma and K. Van Dam, Trends Biochem. Sci., 1 (1976) 16.
4. E. Racker, Annu. Rev. Biochem., 46 (1977) 1006.
5. F.J.R.M. Nieuwenhuis, A.A.M. Thomas and K. Van Dam, Trans. Biochem. Soc., 2 (1974) 512.
6. M. Yoshida, N. Sone, H. Hirata and Y. Kagawa, J. Biol. Chem., 250 (1975) 7910.
7. R.A. Alberty, J. Biol. Chem., 243 (1968) 1337.
8. P.D. Boyer, B. Chance, L. Ernster, P. Mitchell, E. Racker and E.C. Slater, Annu. Rev. Biochem., 46 (1977) 955.
9. P.B. Garland, Symp. Soc. Gen. Microbiol., 27 (1977) 1.
10. R.J.P. Williams, in: Electron Transport and Energy Conservation, eds. J.M. Tager, S. Papa, E. Quagliarriello and E.C. Slater (Bari, Adriatica, 1970) p. 7.
11. F.M. Harold, Bacteriol. Rev., 36 (1972) 172.
12. F.M. Harold, Curr. Top. Bioenerg., 6 (1977) 84.
13. B.A. Haddock and C.W. Jones, Bacteriol. Rev., 41 (1977) 47.
14. P. Mitchell, Nature (London), 191 (1961) 144.
15. P. Mitchell, Symp. Soc. Gen. Microbiol., 20 (1970) 121.
16. P. Mitchell, FEBS Lett., 43 (1974) 189.

17. R.J.P. Williams, FEBS Lett., 53 (1975) 123.
18. R.J.P. Williams, Trans. Biochem. Soc., 5 (1977) 29.
19. P.D. Boyer, Annu. Rev. Biochem., 46 (1977) 957.
20. E.C. Slater, Annu. Rev. Biochem., 46 (1977) 1015.
21. P.D. Boyer, in: Dynamics of Energy Transducing Membranes, eds. L. Ernster R.W. Estabrook and E.C. Slater (Elsevier, Amsterdam, 1974) p. 289.
22. E.C. Slater, in Ref. 21, p. 1.
23. J.W. De Pierre and L. Ernster, Annu. Rev. Biochem., 46 (1977) 201.
24. E. Racker, Trends Biochem. Sci., 1 (1976) 244.
25. M.E. Pullman, H.S. Penefsky, A. Datta and E. Racker, J. Biol. Chem., 235 (1960) 3322.
26. H.S. Penefsky, M.E. Pullman, A. Datta and E. Racker, J. Biol. Chem., 235 (1960) 3330.
27. R.B. Beechey and K.S. Cattel, Curr. Topics Bioenerg., 5 (1973) 305.
28. E. Racker, D.D. Tyler, R. Estabrook, T.E. Conover, D.F. Parsons and B. Chance, in: Oxidases and Related Redox Systems, eds. T.E. King, H.S. Mason and M. Morrison (Wiley, New York, 1964) Vol. 2, p. 1077.
29. R. Serrano, B.I. Kanner and E. Racker, J. Biol. Chem., 251 (1976) 2453.
30. Y. Kagawa, A. Kandrach and E. Racker, J. Biol. Chem., 248 (1973) 676.
31. Y. Kagawa and E. Racker, J. Biol. Chem., 241 (1966) 2461, 2467 and 2475.
32. N. Sone, M. Yoshida, H. Hirata and Y. Kagawa, J. Biol. Chem., 250 (1975) 7917.
33. E. Racker and L.L. Horstman, J. Biol. Chem., 242 (1967) 2547.
34. A. Tzagoloff, D.H. Maclennan and K.H. Byington, Biochemistry, 7 (1968) 1596.
35. G. Toson, A.R. Contessa and A. Bruni, Biochem. Biophys. Res. Commun., 48 (1972) 341.
36. E. Racker, in: Electron Transfer Chains and Oxidative Phosphorylation, eds. E. Quagliariello, S. Papa, F. Palmieri, E.C. Slater and N. Siliprandi (North-Holland, Amsterdam, 1975) p. 401.
37. D.W. Deters, E. Racker, N. Nelson and H. Nelson, J. Biol. Chem., 250 (1975) 1041.
38. S.J. Ferguson, W.J. Lloyd, M.H. Lyons and G.K. Radda, Eur. J. Biochem., 54 (1975) 117.
39. B.I. Kanner, R. Serrano, A. Kandrach and E. Racker, Biochem. Biophys. Res. Comm., 69 (1976) 1050.
40. M.E. Pullman and G.C. Monroy, J. Biol. Chem., 238 (1963) 3762.
41. A.F. Knowles and H.S. Penefsky, J. Biol. Chem., 247 (1972) 6624.
42. L.L. Horstman and E. Racker, J. Biol. Chem., 245 (1970) 1336.
43. M. Satre, M.B. De Jerphanion, J. Huet and P. Vignais, Biochim. Biophys. Acta, 387 (1975) 241.
44. E. Ebner and K.L. Maier, J. Biol. Chem., 252 (1977) 671.
45. K. Asami, K. Juntti and L. Ernster, Biochim. Biophys. Acta, 205 (1970) 307.
46. L. Ernster, K. Juntti and K. Asami, J. Bioenerg., 4 (1973) 149.
47. R.J. Van de Stadt, B.L. De Boer, and K. Van Dam, Biochim. Biophys. Acta, 292 (1973) 338.
48. R.J. Van de Stadt and K. Van Dam, Biochim. Biophys. Acta, 347 (1974) 240.
49. A. Tzagoloff and P. Meagher, J. Biol. Chem., 246 (1971) 7328.
50. A. Tzagoloff, M.S. Rubin and M.F. Sierra, Biochim. Biophys. Acta, 301 (1973) 71.
51. A. Tzagoloff, J. Biol. Chem., 245 (1970) 1545.
52. A. Tzagoloff, Curr. Top. Membr. Transp., 2 (1971) 157.
53. K. Takeshige, B. Hess, M. Boehm, H. Zimmerman-Telschow, Hoppe Seyler's Z. Physiol. Chem., 357 (1976) 1605.

54. E. Ebner, Abstr. 9th FEBS Meeting Budapest (1974) Abstr. S6j6.
55. J.C.M. Hofkenscheid and S.L. Bonting, Biochim. Biophys. Acta, 178 (1969) 129.
56. P.D. Bragg and C. Hou, Arch. Biochem. Biophys., 167 (1975) 311.
57. M. Futai, P.C. Sternweis and L.A. Heppel, Proc. Nat. Acad. Sci. U.S.A., 71 (1974) 2725.
58. G. Giordano, C. Riviere and E. Azoulay, Biochim. Biophys. Acta, 389 (1975) 203.
59. R.L. Hanson and E.P. Kennedy, J. Bacteriol., 114 (1973) 772.
60. N. Nelson, B.I. Kanner and D.L. Gutnick, Proc. Nat. Acad. Sci. U.S.A., 71 (1974) 2720.
61. E. Munoz, J.H. Freer, D.J. Ellar and M.R.J. Salton, Biochim. Biophys. Acta, 150 (1968) 531.
62. J.M. Andreu, J.A. Albendea and E. Munoz, Eur. J. Biochem., 37 (1973) 505.
63. E. Munoz, M.R.J. Salton, M.H. Ng and M.T. Schor, Eur. J. Biochem., 7 (1969) 490.
64. M.R.J. Salton and M.T. Schor, Biochem. Biophys. Res. Commun., 49 (1972) 350.
65. T. Higashi, V.K. Kalra, S.H. Lee, E. Bogin and A.F. Brodie, J. Biol. Chem., 250 (1975) 6541.
66. R. Adolfsen and E.N. Moudrianakis, Biochemistry, 10 (1971) 2247.
67. M. Serrahima-Zeigler and H. Monteil, Biochim, Biophys. Acta, 502 (1978) 445.
68. R. Mirsky and V. Barlow, Biochim. Biophys. Acta, 241 (1971) 835.
69. R. Mirsky and V. Barlow, Biochim. Biophys. Acta, 274 (1972) 556.
70. R. Mirsky and V. Barlow, Biochim. Biophys. Acta, 291 (1973) 480.
71. A. Abrams, C. Jensen and D.H. Morris, Biochem. Biophys. Res. Commun., 69 (1976) 804.
72. H.P. Schnebli and A. Abrams, J. Biol. Chem., 245 (1970) 1115.
73. H.P. Schnebli, A.E. Vatter and A. Abrams, J. Biol. Chem., 245 (1970) 1122.
74. A. Abrams and C. Baron, Biochem. Biophys. Res. Commun., 41 (1970) 858.
75. A. Abrams, E.A. Nolan, C. Jensen and J.B. Smith, Biochem. Biophys. Res. Commun., 55 (1973) 22.
76. M.J.R. Salton, Adv. Microbiol. Physiol., 11 (1974) 213.
77. D. Abram, J. Bacteriol., 89 (1965) 855.
78. A. Asano, N.S. Cohen, R.F. Baker and A.F. Brodie, J. Biol. Chem., 248 (1973) 3386.
79. G.A. Gorneva and I.D. Ryabova, FEBS Lett., 42 (1974) 271.
80. C.W. Jones and E.R. Redfearn, Biochim. Biophys. Acta, 143 (1967) 354.
81. H. Monteil, J. Schoun and M. Guinard, Eur. J. Biochem., 41 (1974) 525.
82. J.D. Oppenheim and M.J.R. Salton, Biochim. Biophys. Acta, 298 (1973) 297.
83. M. Futai, J. Memb. Biol., 15 (1974) 15.
84. W.A. Catterall, W.A. Coty and P.L. Pedersen, J. Biol. Chem., 248 (1973) 7427.
85. W.A. Catterall and P.L. Pedersen, J. Biol. Chem., 246 (1971) 4987.
86. G. Vogel and R. Steinhart, Biochemistry, 15 (1976) 208.
87. J.B. Smith and P.C. Sternweis, Biochemistry, 16 (1977) 306.
88. P.C. Steinweis and J.B. Smith, Biochemistry, 16 (1977) 4020.
89. F.J.R.M. Nieuwenhuis and A.R.J. Bakkenist, Biochim. Biophys. Acta, 459 (1977) 596.
90. B. Chance and G.R. Williams, Adv. Enzymol., 17 (1956) 65.
91. B. Chance, FEBS Lett., 23 (1972) 3.
92. W.G. Hanstein, Biochim. Biophys. Acta, 456 (1976) 129.
93. E.C. Slater, Comprehensive Biochem., 14 (1966) 327.
94. G.D. Greville, Curr. Top. Bioenerg., 3 (1969) 1.
95. H.A. Lardy and S.M. Ferguson, Annu. Rev. Biochem., 38 (1969) 991.
96. K. Van Dam and A.J. Meyer, Annu. Rev. Biochem., 40 (1971) 115.
97. H. Baltscheffsky and M. Baltscheffsky, Annu. Rev. Biochem., 43 (1974) 871.

98. D.E. Green, Trends Biochem. Sci., 2 (1977) 113.
99. S.W. Edwards and D. Lloyd, Biochem. J., 162 (1977) 39.
100. D. Lloyd and S.W. Edwards, Biochem. J., 162 (1977) 581.
101. D. Lloyd and S.W. Edwards, Biochem. J., 160 (1976) 335.
102. R.M. Smith, W.H. Peterson and E. McCoy, Antibiot. Chemother., 4 (1954) 962.
103. S. Masamune, J.M. Sehgal, E.E. Van Tamelen, F.M. Strong and W.H. Peterson, J. Am. Chem. Soc., 80 (1958) 6092.
104. E.W. Marty and E. McCoy, Antibiot. Chemother., 9 (1959) 286.
105. M.H. Larson and W.H. Peterson, Appl. Microbiol., 8 (1960) 182.
106. H.A. Lardy, P. Witonsky and D. Johnson, Biochemistry, 4 (1965) 552.
107. W.F. Prouty, R.M. Thompson, H.K. Schnoes and F.M. Strong, Biochem. Biophys. Res. Commun., 44 (1971) 619.
108. R.B. Beechey, V. Williams, C.T. Holloway, I.G. Knight and A.M. Roberton, Biochem. Biophys. Res. Commun., 26 (1967) 339.
109. M. Von Glehn, R. Norrestam, P. Kierkegaard, L. Maron and L. Ernster FEBS Lett., 20 (1972) 267.
110. G.T. Carter, Ph.D. Thesis, Univ. Wisconsin (1976), in: Diss. Abs. (B), 37 (1976) 766.
111. H. Lardy, P. Reed and C-H. C. Lin., Fed. Proc. Fed. Am. Soc. Exp. Biol., 34 (1975) 1707.
112. R.Q. Thompson, M.M. Hoehn and C.E. Higgens, Antimicrob. Agents Chemother. (1961) 474.
113. R. Michel, P. Huet and M. Huet., C. R. Soc. Biol., 158 (1964) 994.
114. H.A. Lardy, J.L. Connelly and D. Johnson, Biochemistry, 3 (1964) 1961.
115. H.A. Lardy and W.C. McMurray, Fed. Proc. Fed. Am. Soc. Exp. Biol., 18 (1959) 269.
116. H.A. Lardy, D. Johnson and W.C. McMurray, Arch. Biochem. Biophys., 78 (1958) 587.
117. C.L. Wadkins and A.L. Lehninger, Proc. Nat. Acad. Sci. U.S.A., 46 (1960) 1576.
118. C.L. Wadkins and A.L. Lehninger, J. Biol. Chem., 238 (1963) 2555.
119. G.F. Azzone and L. Ernster, J. Biol. Chem., 236 (1961) 1510.
120. A. Bruni, S. Luciani, A.R. Contessa and G.F. Azzone, Biochim. Biophys. Acta, 82 (1964) 630.
121. C. Cooper and R.G. Kulka, J. Biol. Chem., 236 (1961) 2351.
122. R.G. Kulka and C. Cooper, J. Biol. Chem., 237 (1962) 936.
123. C.L. Wadkins, Biochem. Biophys. Res. Commun., 7 (1962) 70.
124. G.P. Brierley, E. Bachmann and D.E. Green, Proc. Nat. Acad. Sci. U.S.A., 48 (1962) 1928.
125. J.B. Chappell, G.D. Greville and K.E. Bicknell, Biochem. J., 84 (1962) 61 P.
126. J.B. Chappell and G.D. Greville, Nature (London), 183 (1959) 1525.
127. J.B. Chappell and G.D. Greville, Biochim. Biophys. Acta, 38 (1960) 483.
128. J.B. Chappell and G.D. Greville, Nature (London), 190 (1961) 502.
129. R. Johnson, S. Scharf and R.S. Criddle, Biochem. Biophys. Res. Commun., 77 (1977) 1361.
130. Y. Kagawa and E. Racker, J. Biol. Chem., 241 (1966) 2461 and 2467.
131. Y. Kagawa and E. Racker, J. Biol. Chem., 241 (1966) 2475.
132. D.H. MacLennan and J. Asai, Biochem. Biophys. Res. Commun., 33 (1968) 441.
133. D.H. MacLennan and A. Tzagoloff, Biochemistry, 7 (1968) 1603.
134. A.F. Knowles, R.J. Guillory and E. Racker, J. Biol. Chem., 246 (1971) 2672.
135. R.E. Jeacocke, D.F. Niven and W.A. Hamilton, Biochem. J., 127 (1972) 57 P.
136. W.A. Hamilton, Adv. Microbiol. Physiol., 12 (1975) 1.
137. P. Scholes and P. Mitchell, J. Bioenerg., 1 (1970) 309.

138. P. Mitchell, FEBS Lett., 33 (1973) 267.
139. P.C. Hinkle and L.L. Horstman, J. Biol. Chem., 246 (1971) 6024.
140. P. Mitchell, Fed. Proc. Fed. Am. Soc. Exp. Biol., 26 (1967) 1370.
141. S. Papa, F. Guerrieri, L. Rossi-Bernadi and J.M. Tager, Biochim. Biophys. Acta, 197 (1970) 100.
142. F.M. Harold, J.R. Baarda, C. Baron and A. Abrams, J. Biol. Chem., 244 (1969) 2261.
143. H. Koboyashi and A. Anraku, J. Biochem. (Tokyo), 71 (1972) 387.
144. M.P. Roisin and A. Kepes, Biochim. Biophys. Acta, 305 (1973) 249.
145. D.R. House and L. Packer, J. Bioenerg., 1 (1970) 273.
146. S. Papa, F. Guerrieri, M. Lorussa and E. Quagliariello, FEBS Lett., 10 (1970) 295.
147. P. Scholes, P. Mitchell and J. Moyle, Eur. J. Biochem., 8 (1969) 450.
148. K. Altendorf, F.M. Harold and R.D. Simoni, J. Biol. Chem., 249 (1974) 4587.
149. R.K. Enns and R.S. Criddle, Arch. Biochem. Biophys., 182 (1977) 587.
150. R.S. Criddle, C. Arulandan, T. Edwards, R. Johnson and S. Schaaf, in: Genetics and Biogenesis of Chloroplasts and Mitochondria, eds. Th. Buecher, W. Neuport and W. Sebald (North-Holland, Amsterdam, 1976) p. 151.
151. G. Zimmer, Biochim. Biophys. Acta, 461 (1977) 268.
152. M.A. Marahiel, G. Imam, P. Nelson, N.J. Pieniazek, P.P. Stepien and H. Kuntzel, Eur. J. Biochem., 76 (1977) 345.
153. C.M. Lazarus, Mol. Gen. Genet., 156 (1977) 303.
154. R.T. Rowlands and G. Turner, Mol. Gen. Genet., 154 (1977) 311.
155. A. Tzagoloff, A. Akai, and F. Foury, FEBS Lett., 65 (1976) 391.
156. A. Brunner, A. Tuena de Cobos and D.E. Giffiths, Mol. Gen. Genet., 152 (1977) 183.
157. D.E. Griffiths, R.L. Houghton, W.E. Lancashire and P.A. Meadows, Eur. J. Biochem., 51 (1975) 393.
158. D.L. Edwards and B.W. Ungar, J. Biol. Chem., 253 (1978) 4254.
159. B. Bulos and E. Racker, J. Biol. Chem., 243 (1968) 3891.
160. H. Schmitz, S.D. Jubinski, I.R. Hooper, K.E. Crook, K.E. Price and J. Lein, J. Antibiot. Ser. A, 18 (1965) 82.
161. H. Schmitz, S.B. Deak, K.E. Crook and R. Hooper, Antimicrob. Agents Chemother., (1963) 89.
162. S. Masamune, J.M. Sehgal, E.E. Van Tamelen, E.E. Strong and W.H. Petersen, J. Am. Chem. Soc., 80 (1958) 6092.
163. P. Walter, H.A. Lardy and D. Johnson, J. Biol. Chem., 242 (1967) 5014.
164. A. Rhodes, K.H. Fantes, B. Boothroyd, M.P. McGonagle and R. Crosse, Nature (London), 192 (1961) 952.
165. M. Brufani, W. Keller-Schierlein, W. Loffler, I. Mansperger and H. Zahner, Helv. Chim. Acta, 51 (1968) 1293.
166. M. Brufani, L. Cellai, C. Musu and W. Keller-Schierlein, Helv. Chim. Acta, 55 (1972) 2329.
167. M. Brufani, S. Cerrini, W. Fedeli, C. Musu, L. Cellai and W. Keller-Schierlein, Experentia, 27 (1971) 604.
168. P. Langcake, R.B. Beechey, C.R. Lindop, S.G.A. Wickens, D.P. Leworthy, D.E. Wiggins and J.M. Broughall, Trans. Biochem. Soc., 2 (1974) 202.
169. P.E. Linnett, A.D. Mitchell and R.B. Beechey, FEBS Lett., 53 (1975) 180.
170. T. Arai, K. Mikami, T. Fukishima, T. Utsumi and K. Yazawa, J. Antibiot., 26 (1973) 157.
171. T. Arai, K. Fukishima and T. Kuga, in: Recent Advances in Medical and Veterinary

Mycology, 6th Congress International Society for Human and Animal Mycology, ed. K. Iwata (University Tokyo Press, Tokyo, 1975) p. 205.
172. P.W. Reed and H.A. Lardy, J. Biol. Chem., 250 (1975) 3704.
173. T. Lichtor and G.S. Getz, Proc. Natl. Acad. Sci. U.S.A., 75 (1978) 324.
174. K. Ishiguro and T. Arai, Antimicrob. Agents Chemother., 9 (1976) 893.
175. H. Baltscheffsky and M. Baltscheffsky, Acta Chem. Scand., 14 (1960) 257.
176. R.A. Ravizzini, W.I.M. Lescano and R.H. Vallejos, FEBS Lett., 58 (1975) 285.
177. Z. Gromet-Elhanan, Proc. 3rd. Int. Symp. Photosynthesis, ed. M. Avron (Elsevier, Amsterdam, 1975) Vol. 1, p. 791.
178. W. Aumuller, Angew. Chem., 75 (1963) 857.
179. A.G. Schering, Belg. Pat. 610,280 (1962), Chem. Abstr., 57 (1962) 12960.
180. Japan, Kokai 76,125,733 (1976) Chem. Abstr. 86 (1977) 151512.
181. R.B. Beechey, C.T. Holloway, I.G. Knight and A.M. Roberton, Biochem. Biophys. Res. Commun., 23 (1966) 75.
182. S. Papa, F. Guerrieri, M. Lorusso and E. Quagliariello, FEBS Lett., 10 (1970) 295.
183. I.A. Kozlov and V.P. Skulachev, Biochim. Biophys. Acta, 463 (1977) 29.
184. W.E. Lancashire and D.E. Griffiths, Eur. J. Biochem., 51 (1975) 377.
185. A.P. Dawson and M.J. Selwyn, Biochem. J., 152 (1975) 333.
186. C.P. Lee, in: Regulation of Metabolic Processes in Mitochondria, eds. J.M. Taylor, E. Quagliariello and E.C. Slater (Elsevier, Amsterdam, 1966), p. 218.
187. R.B. Beechey, Trans. Biochem. Soc., 2 (1974) 466.
188. M.P. Roisin and A. Kepes, Biochim. Biophys. Acta, 305 (1973) 249.
189. A.P. Singh and P.D. Bragg, J. Bacteriol., 119 (1974) 129.
190. K.J. Cattell, I.G. Knight, C.R. Lindop and R.B. Beechey, Biochem. J., 117 (1970) 1011.
191. K.J. Cattell, C.R. Lindop, I.G. Knight and R.B. Beechey, Biochem. J., 125 (1971) 169.
192. A.M. Roberton, C.T. Holloway, I.G. Knight and R.B. Beechey, Biochem. J., 100 (1966) 78P.
193. K. Altendorf and W. Zitzmann, FEBS Lett., 59 (1975) 268.
194. F.S. Stekhoven, R.F. Waitkus and H.B. van Moerkerk, Biochemistry, 11 (1972), 1144.
195. W. Sebald, Biochim. Biophys. Acta, 463 (1977) 1.
196. R.H. Fillinghame, J. Bacteriol., 124 (1975) 870.
197. C. Montecucco and A. Azzi, J. Biol. Chem., 250 (1975) 5020.
198. J.C. Sheenan and G.P. Hess, J. Am. Chem. Soc., 77 (1955) 1067.
199. H.G. Khorani, Chem. Ind. (London), (1955) 1087.
200. H.G. Khorani, Chem. Rev., 53 (1953) 145.
201. K.L. Carraway and D.E. Koshland, Biochim. Biophys. Acta, 160 (1968) 272.
202. K.L. Carraway and R.B. Triplett, Biochim. Biophys. Acta, 200 (1970) 564.
203. T.E. Banks, B.K. Blossey and J. Shaffer, J. Biol. Chem., 244 (1969) 6323.
204. A. Abrams and C. Baron, Biochem. Biophys. Res. Commun., 41 (1970) 858.
205. D. Evans, J. Bacteriol., 104 (1970) 1203.
206. D.L. Gutnick, B.I. Kanner and P.W. Postma, Biochim. Biophys. Acta, 283 (1972) 217.
207. B.I. Kanner and D.L. Gutnick, FEBS Lett., 22 (1972) 197.
208. W.L. Klein and P.D. Boyer, J. Biol. Chem., 247 (1972) 7257.
209. A. Or, B.I. Kanner and D.L. Gutnick, FEBS Lett., 35 (1973) 217.
210. F.R.J.M. Nieuwenhuis, B.I. Kanner, D.L. Gutnick, P.W. Postma and K. Van Dam, Biochim. Biophys. Acta, 325 (1973) 62.
211. D.L. Feinstein and R.J. Fisher, Biochem. J., 167 (1977) 497.
212. J.F. Hare, Biochem. Biophys. Res. Commun., 66 (1975) 1329.

213. R.H. Fillinghame, Fed. Proc. Fed. Am. Soc. Exp. Biol., 34 (1975) 577.
214. L. Patel and H.R. Kaback Biochemistry, 15 (1976) 2741.
215. K. Altendorf, FEBS Lett., 73 (1977) 271.
216. F.M. Harold, E. Pavlasova and J.R. Baarda, Biochim. Biophys. Acta, 196 (1970) 235.
217. C. Baron and A. Abrams, J. Biol. Chem., 246 (1971) 1542.
218. A. Abrams, J.B. Smith and C. Baron, J. Biol. Chem., 247 (1972) 1484.
219. D.J. Clarke and J.G. Morris, Biochem. J., 154 (1976) 725.
220. S.J. Gutowski and H. Rosenberg, Biochem. J., 160 (1976) 813.
221. B.I. Kanner and D.L. Gutnick, J. Bacteriol., 111 (1972) 287.
222. G. B. Cox and F. Gibson, Biochim. Biophys. Acta, 346 (1974) 1.
223. B.I. Kanner, N. Nelson and D.L. Gutnick, Biochim, Biophys. Acta, 396 (1975) 347.
224. P. Friedl, B.I. Schmid and H.U. Schairer, Eur. J. Biochem., 73 (1977) 461.
225. R.D. Simoni and A. Shandell, J. Biol. Chem., 250 (1975) 9421.
226. G.J.N. van der Kerk and J.G.A. Luitjen, J. Appl. Chem., 4 (1954) 314.
227. R.C. Poller, Chemistry of Organotin Compounds, (Academic Press, New York, 1970).
228. K.R.S. Ascher and S. Nissim, Int. Pest Control, 7 (1965) 21.
229. M.S. Blum and J. Prat, J. Econ. Entomol., 53 (1960) 444.
230. G.R. Peiper and J.E. Casida, J. Econ. Entomol., 58 (1965) 392.
231. W.N. Aldridge and J.E. Cremer, Biochim. J., 61 (1955) 406.
232. R.L. Williams and R.L. Metcalf, Science, 158 (1967) 1694.
233. W.P. Neumann, The Organic Compounds of Tin, (J. Wiley, London, 1970) p. 242.
234. R.J. Zedler and C.B. Beiter, Soap and Chemical Specialities (1962) 75.
235. L. Gruen, Gesundheitw. Disinfek., 58 (1966) 81.
236. J.M. Barnes and H.B. Stoner, Br. J. Ind. Med., 15 (1958) 15.
237. H.B. Stoner, Brit. J. Ind. Med., 23 (1966) 222.
238. H.B. Stoner, J.M. Barnes and J.M. McDuff, Br. J. Pharmacol., 10 (1955) 16.
239. J.M. Barnes and L. Magos, Organomet. Chem. Rev., 3 (1968) 137.
240. K. Nakamura and S. Fukishini, Takamine Kenkushno Nempo, 13 (1961) 245 (Chem. Abstr., 63 (1965) 4880).
241. A.S. Watling-Payne and M.J. Selwyn, FEBS Lett., 58 (1975) 57.
242. M.S. Rose and W.N. Aldridge, Biochem. J., 106 (1968) 821.
243. M.S. Rose, Biochem. J., 111 (1969) 129.
244. M. Stockdale, A.P. Dawson and M.J. Selwyn, Eur. J. Biochem., 15 (1970) 342.
245. A. Tzagoloff, K.H. Byington and D.H. Maclennan, J. Biol. Chem., 243 (1968) 2405.
246. W.N. Aldridge and B.W. Street, Biochem. J., 118 (1970) 171.
247. M.J. Selwyn, S.J. Dunnet, R.D. Philo and A.P. Dawson, Biochem. J., 127 (1972) 66 P.
248. D.E. Griffiths and R.L. Houghton, Eur. J. Biochem., 46 (1974) 157.
249. K. Cain, W.E. Lancashire and D.E. Griffiths, Trans. Biochem. Soc., 2 (1974) 215.
250. N. Sone and B. Hagihara, J. Biochemistry (Tokyo), 56 (1964) 151.
251. R.T. Crane, I.L. Sun and F.L. Crane, J. Bacteriol., 122 (1975) 686.
252. A.L. Tappel, Biochem. Pharmacol., 3 (1960) 289.
253. H.J. Harmon and F.L. Crane, Biochem. Biophys. Res. Commun., 59 (1974) 326.
254. I.L. Sun, D.C. Phelps and F.L. Crane, FEBS Lett., 54 (1975) 253.
255. F. Gibson, G.B. Cox, J.A. Downie and S. Radik, Biochem. J., 164 (1977) 193.
256. G.B. Cox, F.L. Crane, J.A. Downie and J. Radik, Biochim. Biophys. Acta, 462 (1977) 113.
257. D.C. Phelps, and F.L. Crane, Biochem. Biophys. Res. Commun., 61 (1974) 671.
258. H.J. Harmon and F.L. Crane, Biochim. Biophys. Acta, 440 (1976) 45.

259. D.C. Phelps, K. Nordenbrand, B.D. Nelson and L. Ernster, Biochem. Biophys. Res. Commun., 63 (1975) 1005.
260. C.L. Baldwin, L.C. Weaver, R.M. Brooker, T.N. Jacobsen, C.E. Osborne and H.A. Nash, Lloydia, 27 (1964) 88.
261. R.B. Beechey, V. Williams, C.T. Holloway, I.G. Knight, and A.M. Roberton, Biochem. Biophys. Res. Commun., 26 (1967) 339.
262. A.M. Roberton, C.T. Holloway, I.G. Knight and R.B. Beechey, Biochem. J., 108 (1968) 445.
263. T-M. Chang and H.S. Penefsky, J. Biol. Chem., 248 (1973) 2746.
264. W.A. Catterall and P.L. Pedersen, J. Biol. Chem., 247 (1972) 7969.
265. C.P. Lee and L. Ernster, Eur. J. Biochem., 3 (1968) 391.
266. G. Lenaz, Biochem. Biophys. Res. Commun., 21 (1965) 170.
267. H.A. Lardy and C-H Lin, *in*: Inhibitors: Tools for Cell Research, ed. T.H. Buecher (Springer-Verlag, New York, 1969) p. 279.
268. R. Adolfsen and E.N. Moudrianakis, Biochemistry, 15 (1976) 4163.
269. A.F. Knowles and H.S. Penefsky, J. Biol. Chem., 247 (1972) 6617.
270. J.L.M. Muller, J. Rosing and E.C. Slater, Biochim. Biophys. Acta, 462 (1977) 422.
271. R.A. Yeates, Biochim. Biophys. Acta, 333 (1974) 173.
272. R.M. Bertina, P.I. Schrier and E.C. Slater, Biochim. Biophys. Acta, 305 (1973) 503.
273. R.J. Van de Stadt, K. Van Dam and E.C. Slater, Biochim. Biophys. Acta, 347 (1974) 224.
274. G.J. Verschoor, P.R. Van Der Sluis and E.C. Slater, Biochim. Biophys. Acta, 462 (1977) 438.
275. E. Agsteribbe, M. Douglas, E. Ebner, T.Y. Koh and G. Schatz; in Genetics and Biogenesis of Chloroplasts and Mitochondria, eds. Th. Buecher. W. Neuport and W. Sebald, (North-Holland, Amsterdam, 1976) p. 135.
276. M.G. Douglas, Y. Koh, M.E. Docktor and G. Schatz, J. Biol. Chem., 252 (1977) 8333.
277. P.W. Reed and H.A. Lardy, J. Biol. Chem., 250 (1975) 3704.
278. H.A. Lucero, W.I.M. Lescano and R.H. Vallejos, Arch. Biochem. Biophys., 186 (1978) 9.
279. H.A. Lucero, R.A. Ravizzini and R.H. Vallejos, FEBS Lett., 68 (1976) 141.
280. A.J. Boulton, P.B. Ghosh and A.R. Katritzky, J. Chem. Soc. Ser. B., (1966) 1004.
281. S.J. Ferguson, W.J. Lloyd and G.K. Radda, FEBS Lett., 38 (1974) 234.
282. A.A. Aboderin, E. Boedefeld and P.L. Luisi, Biochim. Biophys. Acta, 328 (1973) 20.
283. S.J. Ferguson, W.J. Lloyd, M.H. Lyons and G.K. Radda, Eur. J. Biochem., 54 (1975) 117 and 127.
284. J.H. Verheijen, P.W. Postma and K. Van Dam, Biochim. Biophys. Acta, 502 (1978) 345.
285. S.J. Ferguson, P. John, W.J. Lloyd, G.K. Radda and F.R. Whatley, Biochim. Biophys. Acta, 357 (1974) 457.
286. N.C. Price, M. Cohn and R.H. Schirmer, J. Biol. Chem., 250 (1975) 644.
287. St. Metel'skii and I.A. Kozlow, Dokl. Acad. Sci. U.S.S.R. (Biochemistry) 219 (1974) 594.
288. S.J. Ferguson, W.J. Lloyd, G.K. Radda and E.C. Slater, Biochim. Biophys. Acta, 430 (1976) 189.
289. S. Rangaswami, K. Sambamurthy and K. Mallaya-Sastry, Proc. Ind. Acad. Sci. Sect. A, 56 (1962) 239.
290. E.W. Underhill, J.E. Watkin and A.C. Neish, Can. J. Biochem. Physiol., 35 (1957) 219.
291. D.R. Lang and E. Racker, Biochim. Biophys. Acta, 333 (1974) 180.
292. K.K. Jain, D.V. Kohli and R.K. Upadhayay, Indian Drug Pharm. Ind., 11 (1976) 30.

293. I. Beladi, R. Pusztari, I. Musci, M. Bakay and M. Gabar, Ann. N.Y. Acad. Sci., 284 (1977) 358.
294. G. Rodney, A.L. Swanson, L.M. Wheeler, G.N. Smith and C.S. Worrel, J. Biol. Chem., 183 (1950) 739.
295. F. Carpenedo, C. Bortignon, A. Bruni and R. Santi, Biochem. Pharmacol., 18 (1969) 1495.
296. Y. Kuriki and E. Racker, Biochemistry, 15 (1976) 4951.
297. Y. Mukahata, S. Nakabayashi and M. Higashida, FEBS Lett., 85 (1978) 215.
298. A.W. Malyan, E.A. Akulova and E.N. Muzafarov, Bioorg. Khim., 3 (1977) 639.
299. M. Futai, P.C. Sternweis and L.A. Heppel, Proc. Nat. Acad. Sci. U.S.A., 71 (1974) 2725.
300. O.O. Orazi, R.A. Corral, J.S.E. Holker and C. Djerassi, J. Org. Chem., 21 (1956) 979.
301. C. Djerassi, H.W. Brewer, H. Budzikiewecz, O.O. Orazi and R.A. Corral, J. Am. Chem. Soc., 84 (1962) 3480.
302. C. Djerassi, H.W. Brewer, H. Budzikiewecz, O.O. Orazi and R.A. Corral, Experentia, 18 (1962) 113.
303. O.A. Roveri and R.H. Vallejos, Biochim. Biophys. Acta, 333 (1974) 187.
304. R.J. Guillory, Biochim. Biophys. Acta, 89 (1964) 197.
305. R.B. Fisher and R.J. Guillory, Biochim. Biophys. Acta, 162 (1968) 182.
306. E.C. Slater and H.F. Ter Weile, *in*: Inhibitors: Tools in Cell Research, ed. Th. Bucher and H. Sies (Springer Verlag, New York, 1969) p. 258.
307. G. Schatz, H.S. Penefsky and E. Racker, J. Biol. Chem., 242 (1967) 2552.
308. F.M. Harold, J.R. Baarda, C. Baron and A. Abrams, Biochim. Biophys. Acta, 183 (1969) 129.
309. S. Papa, M. Tuena de Gomez-Puyou and A. Gomex-Puyou, Eur. J. Biochem., 55 (1975) 1.
310. M. Tuena de Gomez-Puyou, A. Gomez-Puyou and M. Beigel, Arch. Biochem. Biophys., 173 (1976) 326.
311. M. Tuena de Gomez-Puyou, A. Gomez-Puyou and M. Salmon, Biochim. Biophys. Acta, 461 (1977) 101.
312. G.E. Davies, J. Francis, A.R. Martin, F.L. Rose and G. Swain, Br. J. Pharmacol., 9 (1954) 192.
313. R.M. Calman and J. Murray, Br. Med. J., July (1956) 200.
314. C.H. Lawrence, J. Am. Pharm. Assoc. Sci. Ed., 49 (1960) 731.
315. W.B. Hugo, J. Appl. Bacteriol., 30 (1967) 17.
316. W.B. Hugo and A.R. Longworth, J. Pharm. Pharmacol., 16 (1964) 655.
317. W.B. Hugo and A.R. Longworth, J. Pharm. Pharmacol., 18 (1964) 569.
318. R.N. Rye and D. Wiseman, J. Pharm. Pharmacol., 16 (1964) 516.
319. R.N. Rye and D. Wiseman, J. Pharm. Pharmacol., 17 (1965) 294.
320. D. Daltrey and W.B. Hugo, Microbios, 11 (1974) 113.

Progress in Medicinal Chemistry — Vol. 16, edited by G.P. Ellis and G.B. West

5 Molecular Interactions at the Cholinergic Receptor in Neuromuscular Blockade

J.B. STENLAKE, D.Sc., Ph.D., C. Chem., F.P.S., F.R.I.C., F.R.S.E.

Department of Pharmaceutical Chemistry, University of Strathclyde, Glasgow G1 1XW, Scotland

INTRODUCTION

NEUROMUSCULAR TRANSMISSION

The general pattern of events resulting in the transmission of nervous impulses at the neuromuscular junction is now reasonably well understood [1—4].

Briefly, receipt of the nerve impulse at the axon terminal promotes Ca^{2+}-activated fusion of acetylcholine storage vesicles with the terminal membrane [5,6], releasing acetylcholine into the synaptic cleft. Acetylcholine, so released, combines with specific lipoprotein receptors on the post-junctional membrane. Agonist-receptor combination is immediately followed by changes in receptor conformation [2], with the opening of ion channels and, in consequence, a massive flow of Na^+ and K^+ ions across the muscle membrane. The inward flow of Na^+ ions is much greater and faster than the outward flow of K^+ ions, resulting in a net inward flow of some 3000 univalent cations per acetylcholine molecule released [7]. This surge in membrane conductance [8,9] and its rapid decay all within approx. 0.5 msec, causes the equally rapid fall (depolarisation) and recovery of membrane potential which sets off muscle contraction.

APPROACHES TO RECEPTOR-INTERACTION STUDIES

Knowledge of the essential molecular features of the acetylcholine receptor is still scanty. Isolation of purified receptor proteins by affinity chromatography has paved the way for detailed structural studies. The effect of antagonists on their quaternary structure provides an insight into one possible mechanism to explain ion transfer across the post-junctional membrane. Alternative concepts of the ion-transfer mechanism and its blockade must rest on the detection of specific molecular features of the receptor protein essential to activity. Substantial progress can be reported.

The relatively high proportion of acidic amino acid residues in purified receptor proteins is consistent with the hypothesis that the cation-binding receptor subsite consists of an aspartate or glutamate residue. Likewise, the presence of a disulphide group at the receptor site which is implicated in agonist activity is firmly established. Interest in its role in agonist and antagonist activity, which is still very much a matter for speculation, is heightened by the presence of multiple disulphide bonds, which are essential to retention of both tertiary structure and neuromuscular blocking activity of the snake neurotoxins.

Yet another facet of this expanding picture of the acetylcholine receptor comes from stereochemical studies of agonist and antagonist activity at the acetylcholine receptor. These suggest converse stereochemical requirements for fit at the receptor and the active site of acetylcholinesterase and provide a logical explanation of observed optimum stereochemical requirements for non-depolarising neuromuscular blocking agents related to tubocurarine.

The present review sets out to examine the present state of knowledge of the

cholinergic receptor and its molecular interactions in neuromuscular blockade in an attempt to correlate conclusions based on these separate, but convergent, experimental approaches.

ACETYLCHOLINE RECEPTOR STUDIES

RECEPTOR PROTEINS

Considerable strides have been made in recent years in the isolation and characterisation of purified acetylcholine receptor proteins. These studies have been greatly assisted by the availability of fish electric tissue, by increasing knowledge of snake venom neurotoxins, and by the development of affinity chromatography. The value of studies on electric tissues from the giant South American freshwater eel, *Electrophorus electricus*, and from the giant electric ray, *Torpedo marmorata*, lies in their origin in embryonic tissue similar to that which gives rise to skeletal muscle. In consequence, fully developed electric tissue consists of cells (electroplaques), which are innervated and stimulated by acetylcholine and respond to acetylcholine antagonists in a way which closely resembles that of mammalian neuromuscular junctions. Fish electric tissue, therefore, represents a highly concentrated source of receptor protein for experiment, despite some important differences between electric tissue and mammalian receptors which may well affect the interpretation of results. Thus, it is of particular significance that the density of receptors in the *Torpedo* species is such that ion flow following stimulation occurs exclusively through channels in the acetylcholine receptor itself. In contrast, receptor density in *Electrophorus electricus* tissue is much lower, so that its response to stimulation resembles that of mammalian muscle more closely, in that acetylcholine-receptor association merely triggers the opening of ion channels which appear to be situated for the most part, if not entirely, in the non-receptor protein components of the post-synaptic membrane [10].

Numerous attempts have been made in recent years to isolate and purify cholinergic receptor proteins [11—13]. All the more recent studies have been based on the use as a marker in the isolation procedures of α-bungarotoxin (αBgt) (radiolabelled with ^{131}I). This toxin from the venom of the Taiwan banded krait, *Bungaris multicinctus*, in common with venoms from other Crotalid and Elapid snakes, is intensely and specifically bound to isolated electroplax tissue [10,12,13], blocking the normal response to acetylcholine. In this way, lipid-soluble receptor protein fractions from *Torpedo marmorata* electric tissue [14], and frog, rat and mouse neuromuscular tissue homo-

genates [15,16] have been isolated by solubilisation with the detergent, Triton-X 100. Gel-filtration elution characteristics of the labelled αBgt-receptor protein complex from *Torpedo marmorata* indicated its molecular weight to be in excess of 240 000. Treatment with sodium dodecyl sulphate further showed that the αBgt-receptor protein complex is capable of dissociation into units of MW about 180 000 and 88 000 respectively. The structure of αBgt is now precisely known [17—19] and its molecular weight established as being 7983, leading to a tentative conclusion that the purified cholinergic receptor protein is probably a tetramer of molecular weight about 320 000, which is capable of dissociation in sodium dodecyl sulphate into a dimer and monomer.

Even more highly purified receptor protein has been obtained by affinity chromatography [20] of similar Triton-X 100 solubilised fractions from *Electrophorus electricus*. Fractionation was accomplished using an analogue of gallamine(1,3-bis(trimethylammoniumethoxy)-4-iodoacetamidobenzene di-iodide) as the affinity agent, linked to Sepharose 2B. Elution with gallamine gave a highly active receptor protein with a specific activity of 5400 nmol of *Naja nigricollis* α-[^{3}H]toxin binding site per gram, and an apparent molecular weight of 230 000 when determined by detergent extraction. Polyacrylamide gel-electrophoresis of this protein after treatment with sodium dodecyl sulphate showed that it dissociates into sub-units of two types of mol. wt. 45 000 and 55 000 respectively [21]. Similar receptor protein fractions isolated using *N. nigricollis* α-[^{3}H]toxin [22] and specific affinity labels [23], also dissociate in sodium dodecyl sulphate to sub-units of mol. wt. 40 000—45 000.

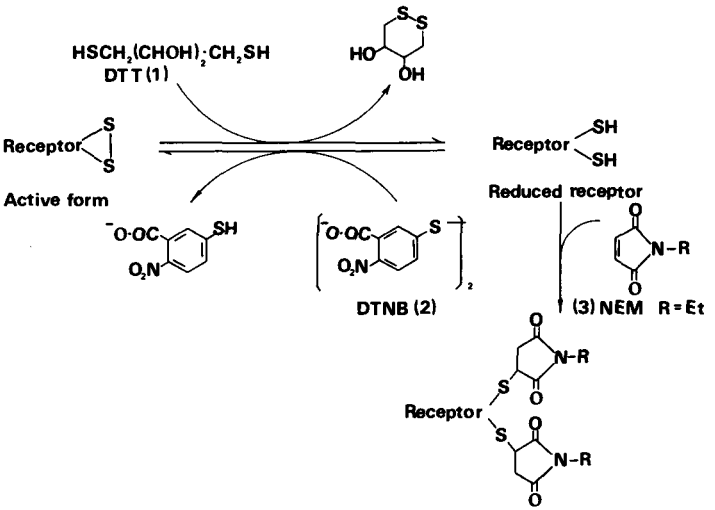
Confirmatory evidence for the presence of two distinctly different types of sub-unit in *Electrophorus electricus* native receptor protein comes from sub-unit cross-linking experiments with suberimidate [24]. These demonstrate the presence of five sub-units, three of mol. wt. 45 000 and two of mol. wt. 54 000 [21] in the associated (quaternary) native receptor protein, which electron microscopy shows are arranged in rosette-like structures. It is of particular interest that native receptor protein when bound either to α-[^{3}H]toxin or to [^{3}H]decamethonium remains capable of partial dissociation, but only into units of mol. wt. about 100 000 or 150 000, thereby demonstrating that these particular inhibitors combine two or more of the lower molecular weight sub-units [21,25,26]. Such inhibition of receptor—sub-unit dissociation suggests a possible mechanism whereby bis-quaternary compounds of appropriate structure may be able to block the formation of ion channels in the receptor. Similar studies show that purified receptor protein from *Torpedo Californica* consists of four distinct types of sub-unit of mol. wt. 39 000, 48 000, 58 000 and 64 000 in association, of which only one (mol. wt. 39 000) binds specific receptor site affinity labels [27].

RECEPTOR-SITE CHEMISTRY

The acetylcholine receptor site is currently held to consist of a cation-binding subsite, with closely-defined stereochemical characteristics, in close proximity to a readily reducible disulphide (—S—S—) group. The existence of the cation-binding subsite is clear although its identity has not been precisely established. In contrast, whilst there is no doubt of the presence of the disulphide bond subsite, its precise function at the receptor site is still uncertain.

The concept of a cation-binding subsite stems from the well-established similarity in primary structure [25,28] and properties [29] of purified acetylcholine receptor protein and acetylcholinesterase. In particular, the consistently high proportion of glutamate (*ca.* 10 mol %) and aspartate (11.5—12 mol %) residues in purified receptor proteins from various *Torpedo* species and *Electrophorus electricus* [25,28] lends weight to the experimentally-unsupported conclusion, that the anionic cation-binding subsites consist of aspartate or glutamate carboxylate groups, as in acetylcholinesterase [30,31].

Inactivation of the cholinergic receptor by heavy metals, first noted some twenty or more years ago [32], provided the first hint of the presence of either sulphydryl (—SH) or the chemically-related disulphide (—S—S—) group at the receptor site. Firm evidence for the disulphide bond subsite came from the



Scheme 5.1

work of Karlin and Bartels [33], which showed that electroplax response to acetylcholine and other agonists in *Electrophorus electricus* is specifically inhibited by prior reaction with the disulphide reducing agent, dithiothreitol (DTT, 1) [34]. Moreover, not only is this inhibition completely reversed by treatment with the sulphydryl oxidising agent, 5,5′-dithiobis(2-nitrobenzoate) (DTNB, 2) [35], but reversal is blocked and acetylcholine inhibition sustained by addition of the sulphydryl blocking agent, *N*-ethylmaleimide (NEM, 3, R = Et).

Experiments with 4-(*N*-maleimido)phenyltrimethylammonium iodide (MPTA, 3, $R = p\text{-}C_6H_4\cdot N^+Me_3I^-$) [36] and 4-(*N*-maleimido) benzyltrimethylammonium iodide (MBTA, 3, $R = p\text{-}C_6H_4\cdot CH_2N^+Me_3I^-$) [37] established that the reactive disulphide bond of the receptor is in close proximity to the cation-binding subsite. Both compounds are merely competitive inhibitors of native receptors, but both are irreversible inhibitors of DTT-reduced receptors. Moreover, the combination in these compounds of an electroplax depolarising group [38] and a sulphydryl-reactive double bond leads not only to irreversible inhibition of DTT-reduced receptors but also to enhancement of the reaction rate. This rate enhancement reaches a maximum of 1100 times that of *N*-ethylmaleimide in MBTA, in which the intramolecular distance between the SH-reactive double bond and the trimethylammonium group is 12Å.

The depolarising activity of maleimide-based affinity reagents at DTT-reduced receptors increases as the intramolecular distance between the SH-reactive double bond and the trimethylammonium group is reduced from 12Å in MBTA (non-depolarising) through MPTA (11.3Å) to 10.6Å in 3-(*N*-maleimido) phenyltrimethylammonium iodide [37]. Depolarisation of DTT-reduced receptors, however, is most marked in bromoacetylcholine bromide and *p*-nitrophenyl-4-*N,N′*-dimethylaminobenzoate methiodide in which the reactive subsites have intramolecular separations of only 9.4 and 9.0 Å respectively. It may, perhaps, be significant that these molecular dimensions approximate very closely to those determined for decamethonium (9.5Å) in aqueous solution [39].

Reduction and alkylation of the disulphide bond receptor subsite not only inhibits the normal agonist response to acetylcholine but also inhibits other receptor agonists, such as carbamylcholine and tetramethylammonium. In contrast, response to the depolarising neuromuscular blocking agent, decamethonium, is increased by disulphide bond reduction, and decreased by subsequent re-oxidation. Some light is perhaps shed on this latter observation by more recent work [28] which established a significant content of free-SH residues in freshly-prepared de-aerated acetylcholine receptor proteins of

both *Electrophorus electricus* and various *Torpedo* species, approximately equivalent to one free-SH group and five S—S groups per acetylcholine binding site in *T. californica.*

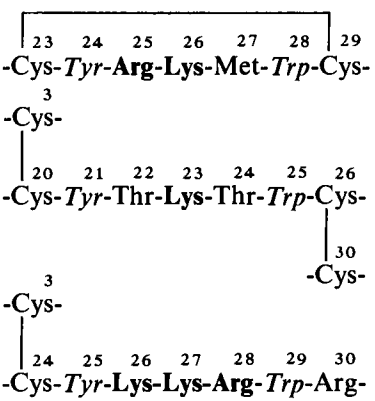
This finding is in keeping with the initial reports by Del Castillo and Katz [32] of receptor inactivation by heavy metals; and also with confirmation of conduction block by phenylmercuric acetate and NEM in frog sartorious muscle and rat phrenic nerve-diaphragm preparations [65]. Some, but not all, of the free-SH groups at the receptor site are active in acetylcholine binding [28] since reaction of either one-third or all free-SH residues with *p*-chloromercuribenzoate reduces maximum ACh binding, though only by 23%. Moreover, it is not clear to what extent agonist response is also inhibited, if at all, by this treatment.

Although the presence of disulphide bonds and free-SH groups in close proximity to the cation-binding subsite are clearly established, evidence for their role in agonist-receptor interaction and in neuromuscular block is purely speculative. It is evident, however, from experiments with the fluorescence probe, 1-anilinonaphthalene-8-sulphonic acid (ANS) on native electroplax tissue, that receptor activity is associated quantitatively with changes in tertiary structure (conformation) [40]. Such evidence is, therefore, consistent with the view advanced by Karlin both that non-covalently bound agonists align themselves with receptor subsites in a way which parallels that of MBTA at DTT-reduced receptors and that binding serves to stabilise the conformation of an adjacent hydrophobic bonding subsite. Evidence for such a hydrophobic site is both circumstantial and tenuous. Nevertheless, it is not inconsistent with the relatively high levels of such amino acids as valine (*ca.* 7 mol %), isoleucine (*ca.* 7.5 mol %) and leucine (*ca.*10 mol %) in purified cholinergic receptor proteins [28], and it has been invoked to explain the lack of depolarising activity in choline, which unlike acetylcholine, would be repelled from such a site [41]. The existence of a hydrophobic bonding site also appears to follow from the increasing neuromuscular blocking potency of alkyltrimethylammonium compounds, as alkyl chain length is increased [42—45].

Even if it is accepted that conformational changes leading to alignment of a hydrophobic bonding subsite are essential for agonist alignment, the molecular mechanism whereby interaction between a disulphide bond and a typical agonist could induce a change in protein confirmation is not clear. In contrast, the inactivation of receptors by heavy metals [32], the confirmation of the presence of free-SH residues in purified receptor proteins [28], and the significance of intact disulphide bonds for maintenance of neurotoxicity of snake venoms, all point towards specific interactions involving well-established sulphydryl-disulphide displacement reactions.

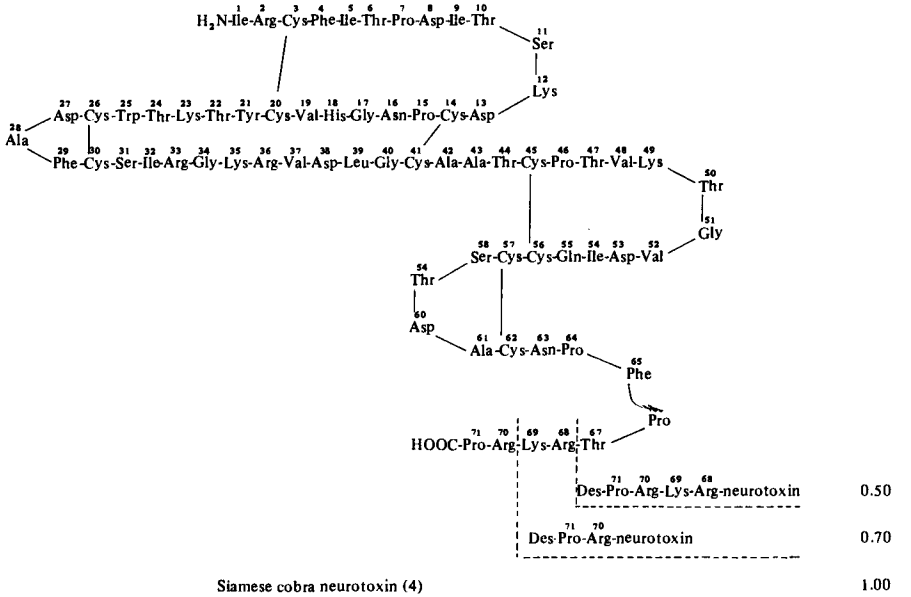
SNAKE NEUROTOXINS

Considerable progress has been made in recent years towards the characterisation of α-bungarotoxin and the various neurotoxic cobra venoms, all of which are potent neuromuscular blocking agents. All are peptides, each of some 61—74 amino acid units. The complete amino acid sequences have now been established for α-bungarotoxin [17—19], for toxin A from Indian cobra (*Naja naja*) venom [46], for East Indian cobra (*Naja naja naja*) and Siamese cobra (*Naja Siamensis 3*) venoms [47], for Formosan cobra (*Naja naja atra*) venom [48], Egyptian cobra (*Naja haje*) venom [49], and Cape cobra (*Naja nivea*) α-, β- and γ-venoms [50—51] showing them to consist of a single chain of 74, 71, 72, 71, 62, 61, 71, 61 and 61 amino acids respectively. A number of these toxins are identical, as in the case of Indian, Siamese and Cape α cobra toxins, and also Egyptian and Cape γ cobra toxins. Several have significant elements of their amino acid sequences in common, and all have similar folded structures which are maintained by similarly placed multiple disulphide cross-links. The longer chain toxins such as α-bungarotoxin and Siamese cobra toxin (4) have five disulphide bridges, while the shorter chain 61 and 62 unit peptides have only four such links. One disulphide bond is also regularly associated with an adjacent tyrosine residue, having tryptophan four residues removed, and one to three basic amino acids forming part of the intervening chain, as in the following sequences.



Additionally, all the neurotoxins are characterised by the possession of a substantial number of lysine and arginine residues, in considerable excess over the number of acidic residues, which like the disulphide bridges have an important bearing on neuromuscular blocking activity. Significantly, many of these basic amino acid residues are grouped in pairs or threes, or alternatively,

spaced some three, four or six units apart to appear side-by-side on adjacent or alternating turns of the helix coils.



Typical arrangements of basic amino acid residues are seen in Siamese cobra venom (4) [47] which has the adjacent pair, H_2N-Ile^{1}-Arg^{2}, the adjacent triplet, Arg^{68}-Lys^{69}-Arg^{70}, and a sub-adjacent triplet, Arg^{33}-Gly-Lys^{35}-Arg^{36}, in addition to the isolated residues, Lys^{12}, Lys^{23}, Lys^{49}. Likewise, α-bungarotoxin has the adjacent pairs, Arg^{25}-Lys^{26}, Lys^{51}-Lys^{52}, Lys^{70}-Arg^{71}, the sub-adjacent pair, Arg^{36}-Gly-Lys^{38}, and the isolated residues, Lys^{38} and Lys^{64} [17].

The significance of the strongly basic characteristics which these amino acids confer on the neurotoxins has been examined by chemical modification of the lysine and *N*-terminal isoleucine residues in Siamese (4) and East Indian cobra venoms (*Table 5.1*). This shows that no single one of these groups is itself essential for the venom's lethal curarising action. Carbamoylation of either the *N*-terminal amino group or one of the lysine residues (undesignated) of Siamese cobra venom reduces relative toxicity by one-third, and all six monoacetylated toxins also show a similar reduction in potency. Sequential blocking of the remaining lysyl residues reduces toxicity still further. Thus, fractions in which the α-amino (H_2N-Ile) and one, two, three, four and five ε-amino (lysyl-NH_2) groups respectively are carbamoylated have relative toxicities of 0.50, 0.33, 0.25, 0.07 and 0.03 that of the native protein. The

toxicity of pentacarbamoylated East Indian cobra toxin, which differs only at residues 28 (Ala in place of Gly) and, significantly, 49 (Arg in place of Lys), is reduced, however, to only 20% compared with 7% for the corresponding pentacarbamoylated Siamese cobra toxin. Similarly, conversion of all five lysyl residues in the Siamese toxin to homoarginine by guanidation, leaving the *N*-terminal isoleucine residue intact reduces toxicity, but only to some 50% that of the native protein. It appears, therefore, that whereas all strongly basic groups confer activity, this is greater in the less space-demanding α-*N*-terminal-NH_2 and ε-lysyl-NH_2 groups than in the somewhat larger guanidino groups of arginine and homoarginine residues.

A substantial element of the neurotoxicity of the longer chain peptides, such as Siamese cobra neurotoxin, however, also lies in the C-terminal amino acid sequence [52]. Thus, incubation of the native toxin with Arthrobacter proteinases gave the des-Pro^{71}-Arg^{70} and des-Pro^{71}-Arg^{70}-Lys^{69}-Arg^{68} peptides with relative toxicities of 0.70 and 0.50 respectively.

The importance of intact disulphide bridges in neurotoxic activity has been clearly demonstrated in a number of experiments by Yang [53,54] on Formosan cobra toxin. Thus, complete reduction of all disulphide bonds to sulphydryl groups with β-mercaptoethanol in Formosan cobra toxin causes

Table 5.1. RELATIVE TOXICITIES OF CARBAMOYL DERIVATIVES OF SIAMESE COBRA NEUROTOXIN [47]

Position[1,2] *and Number of carbamoyl groups*	*Relative toxicity*
None	1.00
α^1	0.67
ε^2	0.67
$\alpha+\varepsilon$	0.50
$\alpha+2\varepsilon$	0.33
$\alpha+3\varepsilon$	0.25
$\alpha+4\varepsilon$	0.07
$\alpha+5\varepsilon$	0.03
$\alpha+4\varepsilon$	0.20[3]

[1] *N*-Terminal α-amino group (H_2N—Ile).
[2] Lysyl ε-amino groups.
[3] East Indian cobra neurotoxin.

almost complete loss of its lethality (only 2.6% of lethality of native toxin remains) with marked changes in its infrared spectrum and optical rotation ($[\alpha]_D^{27}$ −82°; native toxin, $[\alpha]_D^{27}$ −3°). These changes, however, are almost completely reversed by mild pH-dependent air oxidation, which restores full lethality and the physical characteristics of the native toxin [53]. ORD studies on the native toxin showed positive Cotton effects at 233 nm and a mean residue rotation of + 1250°, indicative of a left-handed, α-helical, tertiary structure [54]. Both β-mercaptoethanol-reduced and reduced carboxymethylated-toxin showed a negative trough at 233 nm and a mean residue rotation of −2100, compatible with the formation of a random-coiled conformation, whilst the ORD curve of re-oxidised reduced toxin was identical with that of the native toxin. The CD spectrum of the native toxin also shows a minimum at 285 nm, which is not present in the reduced toxin. This confirms that elements of asymmetry destroyed by reduction are present in the native protein in the environment of aromatic amino acid residues, probably tyrosine in accord with its structure [48]. (See note on p. 286).

It is possible that the primary function of the multiple disulphide bridging characteristic of these neurotoxins is simply the maintenance of a compact globular structure, which by virtue of the orientation of hydrophobic amino acid residues endows the molecular structure with transport characteristics that promote its location in the lipid tissue elements of the neuromuscular junction. An alternative explanation of their significance may, however, lie in the coincidence that disulphide bonds and perhaps, more significantly, free-sulphydryl groups also figure as essential features of the active centre of the acetylcholine receptor protein.

STEREOCHEMISTRY OF RECEPTOR INTERACTIONS

QUANTITATIVE ASPECTS OF ACETYLCHOLINE-RECEPTOR INTERACTION AND ITS TERMINATION BY ACETYLCHOLINESTERASE

Quantitative measurements have shown that receipt of the nerve impulse leads to simultaneous release of some 4.2×10^6 molecules of acetylcholine [55] with the disruption and disappearance of some 200—400 vesicles [56] from each axon terminal. Experiments with radio-labelled α-bungarotoxin, which is irreversibly-bound at neuromuscular junctions, have shown that rat and mouse diaphragms have about 4×10^7 and $2—3 \times 10^7$ acetylcholine binding sites (receptors) per end-plate respectively [57]. These agree with results obtained by autoradiography on mouse diaphragm [58], using the depolarising neuromuscular blocking agent, [^{14}C]decamethonium (7.5×10^7), but they

are some ten times greater than receptor numbers obtained by autoradiography with the non-depolarising blocking agents, [^{14}C]curarine (4×10^6) and [^{14}C]toxiferin (4×10^6). It has, however, also been shown that the normal α-bungarotoxin binding, equivalent to 10^9 receptors per end-plate in frog sartorious muscle, is reduced by some 50% in the presence of tubocurarine [15]. The further reduction of α-bungarotoxin binding in the presence of acetylcholine, as well as tubocurarine, indicates that the α-bungarotoxin receptors are true cholinergic receptors.

In summary, therefore, nerve stimulation leads to the release of some 4×10^6 molecules of acetylcholine at each axon terminal in proximity to some 4×10^7 receptor sites.

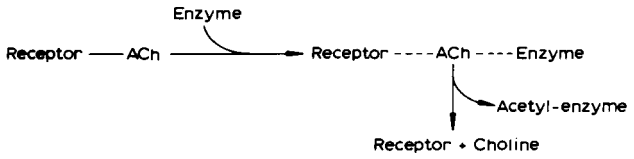
It is widely accepted that enzymatic hydrolysis by acetylcholinesterase, rather than agonist-receptor kinetics, is the rate-controlling process in termination of acetylcholine action at the neuromuscular junction. In accord with this concept, decay of the end-plate current is more rapid than diffusion of acetylcholine out of the synaptic cleft [59]. Also, the action of acetylcholine is prolonged in the presence of cholinesterase inhibitors, such as neostigmine [60,61]. Autoradiographic studies with the anti-cholinesterases, [^{32}P]DFP and [^{14}C]DFP, on mouse diaphragm show the presence of some 2.4×10^7 acetylcholinesterase centres located in specific sites on the surface of the post-junctional membrane facing into the junctional cleft [58]. These acetylcholinesterase centres are present, therefore, in equal number and in close proximity to the acetylcholine receptors.

AGONIST-RECEPTOR AND AGONIST-ESTERASE INTERACTIONS

Although it has been suggested that the binding sites at the acetylcholine receptor and the active site of acetylcholinesterase are structurally and stereochemically identical [62,63], there is now good evidence to show that this is not so. Thus, whilst αBgt binds irreversibly [64] at the acetylcholine receptor, it is not bound by acetylcholinesterase [61]. Similarly, the receptor is susceptible to block by sulphydryl reagents [28,32,65], whereas acetylcholinesterase is unaffected by them. Also, removal of basement membrane material from the synaptic cleft by digestion with collagenase abolishes membrane-bound acetylcholinesterase activity but releases acetylcholinesterase into solution [66], implying that the enzyme is situated in a relatively exposed position and is not a full structural component of the membrane.

Despite the fact that diffusion from the synaptic cleft is not the rate-controlling process [59], it has usually been assumed by implication, rather than by explicit statements or observations to the contrary, that agonist-

receptor dissociation precedes agonist-acetylcholinesterase association. However, experimental observations, such as prolongation of agonist activity in the presence of cholinesterase inhibitors [60,61], do not exclude alignment for esterase binding of acetylcholine molecules through changes in membrane conformation whilst still receptor-bound, leading to hydrolysis, inactivation and release of choline in a series of steps which might be represented as in *Scheme 5.2*.



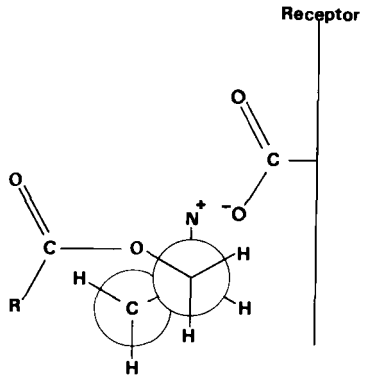
Scheme 5.2

Several factors suggest that this concept is worthy of further examination. Firslty, as already indicated, both receptor and esterase proteins are present in equivalent number in close proximity on the surface of the post-junctional membrane. Secondly, agonist-receptor association releases Ca^{2+} ions from the subsynaptic membrane [67]; these ions are known to activate acetylcholinesterase [68]. Also, agonist-receptor association is accompanied by conformational changes in the receptor protein [2], which might conceivably, at one and the same time, effect enzyme-substrate alignment [69]. More significantly, however, it is axiomatic that an agonist molecule, which is firmly attached to its receptor, can only be approached by its enzymic inactivator at a complementary or opposite face. The stereochemistry of agonist-receptor and antagonist-receptor association should therefore differ from, if not actually mirror, that of agonist-AChE association. There is evidence that this is so in the binding of α-bungarotoxin, in the acetyl α- and β-methylcholines, and in the succinyl-α- and β-methylcholines. Furthermore, receptor stereochemistry, based on that of the more active isomers of these compounds, accords with that observed from the favoured stereochemistry of other neuromuscular blocking agents already described [70,71].

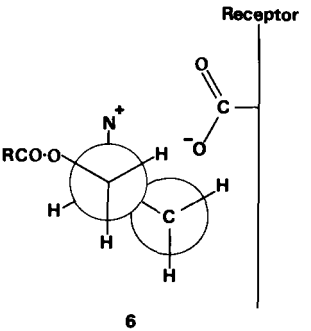
($\pm$)-Acetyl α-methylcholine has approximately half the potency of acetylcholine as an agonist in producing a twitch response in denervated cat gastrocnemius muscle [72]. Since the relative orientation of the acetoxy group and quaternary centre is the same in both isomers, receptor association is hindered by the α-methyl substituent in one of the stereoisomers. Furthermore, if it is accepted that in their receptor and enzymic interactions, they adopt analogous *gauche* conformations to that of acetylcholine in solution [73], it is evident from the Newman projections (5, R = Me) and (6, R = Me)

of the (*R*)- and (*S*)-isomers [78] respectively, that approach to the receptor is probably, though not certainly, more hindered in the (S)-isomer (6), due to the greater effect of the *α*-methyl group in increasing the N^+-receptor amine subsite distance in that isomer.

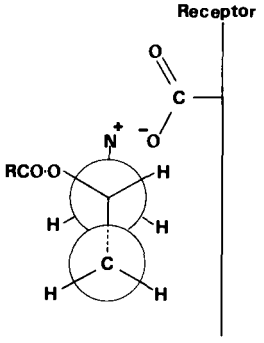
This effect is only apparent in receptor approach at the *rear lower right* octant when the molecule is arbitrarily orientated for reference as in (9) [82], in comparison with acetylcholine (10).



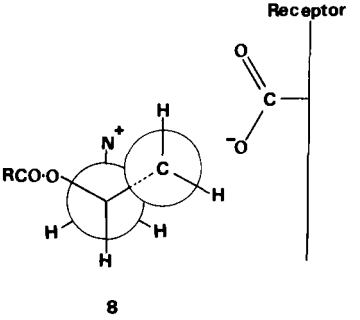
5



6



7

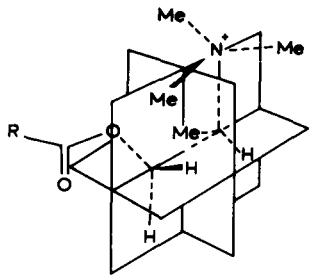


8

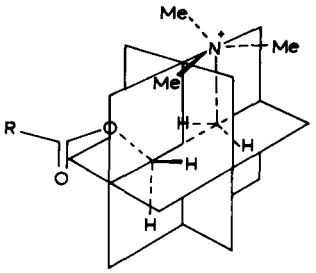
The *β*-methyl substituent in the acetyl *β*-methylcholines (7) and (8) is attached to the acyloxy carbon atom. It is, nevertheless, sufficiently far removed from the carbonyl carbon to exert little more than marginal influence either sterically or electronically upon nucleophilic attack, as for example by the enzyme's serine hydroxyl in hydrolysis as proposed by Krupka [30,31]. It

is, however, capable of exerting steric hindrance to receptor-agonist association and to the approach of the acetylcholinesterase-histidine residue (pK_a 5.5) which acts catalytically in enzyme hydrolysis as hydrogen donor to the carbonyl oxygen and hydrogen acceptor from the serine hydroxyl [31].

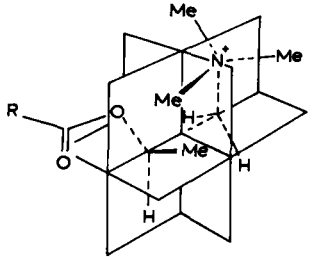
Since (±)-acetyl β-methylcholine has only 1% of the agonistic effect of acetylcholine at the neuromuscular junction [72], drug-receptor association must be even more hindered by the methyl substituent in the β-isomers than is the corresponding α-isomers. Assuming the *gauche* conformations shown in the Newman projections (7, R = Me) and (8, R = Me) for the (*S*)- and (*R*)-isomers respectively, a high degree of hindrance in both isomers is compatible only with agonist-receptor association at an anionic receptor binding site abutting both *front upper* and *lower right* octants when orientated as in (11). The agonistic activities of both α- and β-methylcholines can, therefore, be accommodated only by agonist-receptor anionic binding at the *right hand* face of acetylcholine when similarly orientated as in (10).



9



10



11

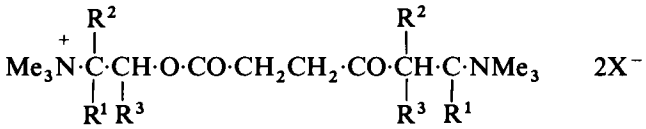
In contrast, whereas (*S*)-(−)-acetyl α-methylcholine (6, R = Me) is hydrolysed by acetylcholinesterase at a rate which is 97% that for acetylcholine, enzymic hydrolysis of (*R*)-(+)-acetyl α-methylcholine (5, R = Me) proceeds at only 78% of the rate of acetylcholine hydrolysis [74]. These results establish

that agonist-enzyme interaction occurs in the rear *left* octant, though they do not show whether the inhibitory effect of the methyl group in the (*R*)-isomer is due to interference with agonist-enzyme binding or steric inhibition of enzyme hydrolysis. Additionally, however, (*S*)-(+)-acetyl *β*-methylcholine (7, R = Me) is hydrolysed at only 54% of the rate of acetylcholine hydrolysis, whilst its (*R*)-(−)-isomer (8, R = Me), significantly, is not hydrolysed, but instead has weak enzyme-inhibitor activity. The (*S*)-isomer is, therefore, enzyme-bound and is a substrate, whilst on the other hand, the (*R*)-isomer, although enzyme-bound, is capable only of inhibiting the binding and hydrolysis of the natural substrate. These observations are compatible with unhindered agonist-enzyme association with the enzyme's anionic binding site abutting the upper and lower rear *left* octants of the ester combined with steric hindrance to the approach of the enzyme's catalytic histidine residue at the *front* face of the *front upper right* octant of the substrate molecule (10).

It is apparent, therefore, that whereas acetylcholine-receptor association occurs at the molecule's *right hand* face (10, R = Me), agonist-acetylcholinesterase interactions occur at its complementary *left hand* and *front* faces.

SOME ANTAGONIST-RECEPTOR AND ANTAGONIST-ESTERASE INTERACTIONS

The conclusions concerning the stereochemistry of agonist-receptor interactions are supported by the relative efficacies of the receptor antagonist, succinylcholine (12, $R^1 = R^2 = R^3 = H$), and its *α*- and *β*-methyl homologues. Thus, Rosnati [75] has shown that the neuromuscular blocking potency of succinylcholine as measured by rabbit head drop is reduced from an arbitary 100 to a relative potency of 22.5 in (±)-succinylbis-*α*-methylcholine (12, $R^1 = Me$; $R^2 = R^3 = H$), to 6 in (±)-succinylbis-*α*,*α*-dimethylcholine (12, $R^1 = R^2 = Me$; $R^3 = H$), and to < 0.36 in (±)-succinylbis-*β*-methylcholine (12, $R^1 = R^2 = H$; $R^3 = Me$). Studies by Lesser [76,77] of the optically active succinylbis-*α*- and *β*-methylcholines [78] on the cat gastrocnemius muscle preparation (*Table 5.2*) show similar reductions in potency which, likewise, are much greater in the *β*- than in the *α*-isomers.



12

Table 5.2. RELATIVE POTENCIES OF OPTICAL ISOMERS OF SUCCINYLBIS-α- AND β-METHYLCHOLINES [76,77]

Isomer	*Potency (Mol ≡ 1 mol succinylcholine)*
(*S*)-(−)-α	2.6
(*R*)-(−)-α	1.8
(*S*)-(+)-β	890
(*R*)-(+)-β	1200

The reduction in potency in both α-isomers shows that receptor-antagonist association can be hindered in both rear lower octants, but the greater reduction in potency in the (*S*)-(−)-α-isomer (6, $R = Me_3N^+ \cdot CHMe \cdot CH_2 \cdot O \cdot CO \cdot CH_2 \cdot CH_2 -$) implies a preference for close receptor fit at the *rear lower right* octant of the antagonist. The significantly lower potency of the two β-isomers, similarly, shows that receptor-antagonist association is hindered in the *front upper right* and either one or both *front lower* octants. The greater reduction in potency of the (*R*)-(−)-β-isomer (8, $R = Me_3N^+ \cdot CH_2 \cdot CHMe \cdot O \cdot CO \cdot CH_2 \cdot CH_2 -$) implies that receptor approach occurs preferably at the *upper front right* octant of these two antagonists. The relative receptor blocking activities of all four succinylbis-α- and β-methylcholines when considered together can therefore be explained best by antagonist-receptor binding at their *right hand* faces when represented as shown in the projections (5), (6), (7) and (8).

The close parallel between the hydrolysis rates of the corresponding succinylbis- and butyryl-α- and β-methylcholines [74] by human plasma pseudocholinesterase demonstrates that enzymic interactions are controlled by steric parameters other than that of the acyl substituent (*Table 5.3*).

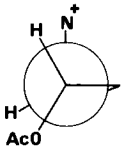
The hydrolysis of (*S*)-(−)-succinylbis-α-methylcholine (6, $R = Me_3N^+ \cdot CHMe \cdot CH_2 \cdot O \cdot CO \cdot CH_2 \cdot CH_2 -$) at virtually the same rate as that of succinylcholine [79], and the hydrolysis of the corresponding (*R*)-(+)-isomer (5, $R = Me_3N^+ \cdot CHMe \cdot CH_2 \cdot O \cdot CO \cdot CH_2 \cdot CH_2 -$) at only some 40% of that rate, therefore, indicate that enzyme interaction occurs at the *left hand* face of both these compounds. The even lower rate of hydrolysis of (*S*)-(+)-succinylbis-β-methylcholine (7, $R = Me_3N^+ \cdot CH_2 \cdot CHMe \cdot O \cdot CO \cdot CH_2 \cdot CH_2 -$) and the zero hydrolysis rate of (*R*)-(−)-succinylbis-β-methylcholine (8, $R = Me_3N^+ \cdot CH_2 \cdot CHMe \cdot O \cdot CO \cdot CH_2 \cdot CH_2 -$), together with the signifi-

Table 5.3. RATES OF HYDROLYSIS OF SUCCINYLBIS-AND BUTYRYL-*α*- AND *β*-METHYLCHOLINES BY HUMAN PLASMA PSEUDO-CHOLINESTERASE [78]

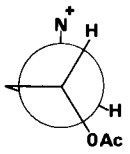
Isomer	*Hydrolysis rates*	
	Succinylbis-methylcholine (Succinylcholine = 100)	*Butyrylmethylcholine (Butyrylcholine = 100)*
(*S*)-(−)-*α*	100	93
(*R*)-(+)-*α*	38	79
(*S*)-(+)-*β*	5	5
(*R*)-(−)-*β*	0	0

cantly greater inhibitory constant of the (*R*)-isomer for inhibition of butyrylcholine hydrolysis ($K_i = 25 \times 10^{-4}$M) compared with that of the (*S*)-isomer ($K_i = 7.5 \times 10^{-4}$M) and succinylcholine ($K_i = 6.0 \times 10^{-4}$M), however, additionally imply that enzymic interaction also occurs at the *front* face of the molecules. Taken together, these hydrolysis and inhibition studies for both sets of molecules support the view that hydrolysis requires attack at the *rear left* and *front* faces of the molecules in contrast to neuromuscular block which involves the *right* and *front* faces.

Neuromuscular blocking potencies and anticholinesterase hydrolysis rates for *cis*- and *trans*-2-acetoxycyclopropyltrimethylammonium iodides are both incomplete and equivocal because of their very low blocking potencies, and because of the departure from the *gauche* conformation of onium and acetoxy groups of acetylcholine. The (−)-*trans* isomer has, however, double the neuromuscular blocking potency, but is hydrolysed at only 60% of the rate of (+)-*trans* isomer [80,81]. Despite the fact that the absolute stereochemistry of these isomers (13 and 14) is not known, these results are consistent with impedence by the cyclopropane ring to antagonist-receptor binding at one face of the molecule and impedence to antagonist-enzyme attack at the opposite face. Neuromuscular blocking potencies only are recorded for the unresolved *cis*-isomer.

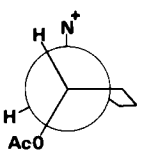


13

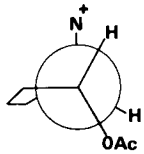


14

Similar conclusions concerning the stereochemical requirements for enzymic hydrolysis can also be drawn from the relative efficiencies of the isomers of 2-dimethylaminocyclopentyl acetate methiodide as substrates for bovine erythrocyte acetylcholinesterase [82]. V_{max} values of 10.1×10^7 mol·min^{-1} for the (1*S*,2*S*)-(−)-*trans*-isomer (15) and 5.4×10^7 mol·min^{-1} for the (1*R*,2*R*)-(+)-*trans*-isomer (16) compared with zero for the (±)-*cis*-isomer, show that enzymic attack at the *left hand* face of the molecule is favoured. Unfortunately, the larger hydrocarbon ring (compared with that of the cyclopropyl compounds 13 and 14) reduces the neuromuscular blocking potencies of all the isomers to negligible levels (frog rectus abdominis muscle) and relative potencies have not been recorded.



15



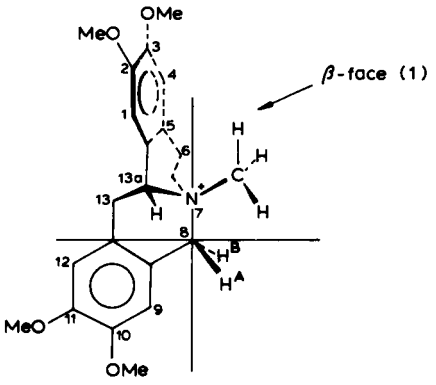
16

In summary, there is a substantial body of evidence to suggest that the receptor protein and acetylcholinesterase have opposite stereochemistry at their respective anionic subsites, which interact with the quaternary centre and α-carbon in the rear octants of both agonists and antagonists. It is equally evident, however, that both receptor and enzyme also have important active centre subsites which interact at the front right octants of agonists and antagonists. Simultaneous attachment to both receptor and enzyme, therefore, is clearly impossible. Nevertheless, the stereochemical evidence is not inconsistent with the idea of agonist (or antagonist) being presented to the enzyme for hydrolysis, whilst still receptor-bound, through a loose conformationally-dependent receptor-agonist-enzyme complex, but there is no evidence that this is so.

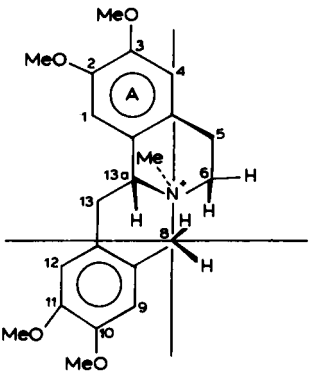
TETRAHYDROISOQUINOLINIUM NEUROMUSCULAR BLOCKING AGENTS

The geometry of antagonist-receptor association at the neuromuscular junction, which emerges from the foregoing stereochemical analysis, conforms with conclusions based on the relative potencies of stereoisomers of 1-benzyltetrahydroisoquinolinium salts [70] and norcoralydine methiodide [71].

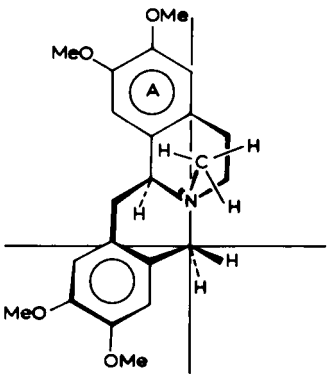
Thus, relative potencies of the norcoralydine methiodides correlate positively with antagonist-receptor association at the *right hand* face of the molecules when orientated about the quaternary centre in a similar way to acetylcholine (10) with respect to the anionic receptor subsite, as represented in (17), (18), (19) and (20) [4]. Potency is highest in (*R*)-*cis*-norcoralydine methiodide (17; molar potency 13.6 relative to tubocurarine = 100) in which only the *N*-methyl and C-8 protons protrude into the *right hand* octants. Potency falls to 4.5 in (*R*)-*trans*-norcoralydine methiodide (18) with C-5 and C-6 methylenes and C-8 protons in *right hand* octants. A further fall in potency to 3.2 occurs in (*S*)-*trans*-norcoralydine methiodide (19) with *N*-methyl protons, C-5 and C-6 methylenes, and C-8 protons in *right hand* octants, whilst potency is least (2.1) in (S)-*cis*-norcoralydine methiodide (20), in which most of rings C and D protrude extensively into right hand octants.



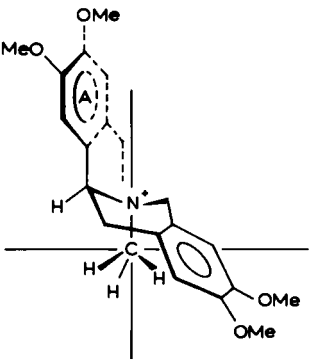
17



18



19



20

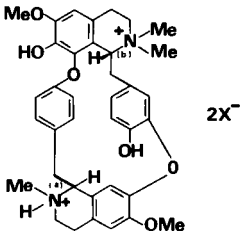
These conclusions have been confirmed by the synthesis of a group of specifically deuterated derivatives of (*R*)-*cis*- and *trans*-norcoralydine in which hydrogen has been replaced by deuterium at key points that would be expected to impinge on the receptor [83]. These studies are based on the concept that the zero point energy of deuterium is less than that of hydrogen [84], and that this leads amongst other effects to a shortening of the van der Waals radius. As a result, molecules which incorporate deuterium in place of hydrogen have a reduced space requirement about the C—D bonds compared with that of the corresponding nondeuterated compounds [85,86]. Whilst deuteration has only marginal effect on the potency of the *trans*-compounds, the *cis*-compounds, in contrast, all showed major potency enhancements (*Table 5.4*) in accord with the concept of drug-receptor subsite interaction at the *front right hand upper* ($N^+—CH_3$), *front right hand lower* ($C_8—H^A$) and *front left hand upper* ($C_{13a}—H$) octants of the *cis*-compounds when viewed as represented in structure (17).

Consideration of the conformation conferred by the two tetrahydropapaverine units in tubocurarine (21) and its stereoisomers in relation to that of (*R*)-*cis*-norcoralydine (17) provides a rational explanation of their relative potencies. Thus, the conformation of the tetrahydroisoquinolinium moiety comprising rings A and B of (*R*)-*cis*-norcoralydine is identical with that of (*S*)-laudanosine methiodide [71]. Hence, it is apparent that only the non-quaternary centre (a) of absolute stereochemistry, *S*, in tubocurarine (21) is able to present itself to the receptor in a like manner to the favoured (*R*)-*cis*-norcoralydine, whereas the quaternary ammonium group at centre (b) has the unfavourable (*S*)-norcoralydine stereochemistry. In contrast, it is the quaternary ammonium group at centre (a) of isotubocurarine (22) [87] which has the favoured (*R*)-norcoralydine conformation, and the non-quaternary centre (b)

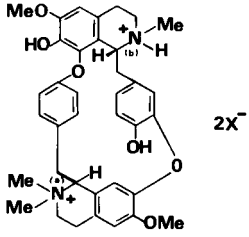
Table 5.4. RELATIVE MOLAR NEUROMUSCULAR BLOCKING POTENCIES OF DEUTERIO-NORCORALYDINES [83]

Compound	*Relative molar potencies*
(*R*)-(+)-*cis*-Norcoralydine methiodide	100
(*R*)-(+)-*cis*-Norcoralydine trideuteriomethiodide	218
(*R*)-(+)-*cis*-[8,8-^{2}H]-Norcoralydine methiodide	222
(*R*)-(+)-*cis*-[8,8-^{2}H]-Norcoralydine trideuteriomethiodide	240
(*R*)-(+)-*cis*-[13,13,13a-^{2}H]-Norcoralydine trideuteriomethiodide	280

which has the unfavourable (*S*)-norcoralydine geometry, in accord with its much higher neuromuscular blocking potency (= 230) relative to that of tubocurarine (= 100).

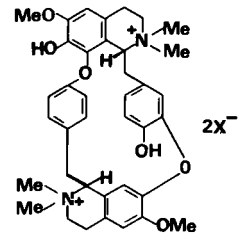


21

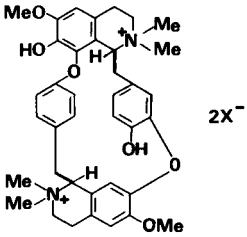


22

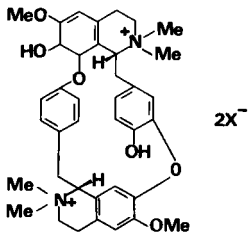
Equally significantly, whilst the enhancement of potency to 290 in the bis-quaternary compound, chondocurarine (23) with identical (*R,S*)-stereochemistry to isotubocurarine (22) is only marginal [88], the bis-quaternary (+)-curarine (24) with the doubly favoured (*S,S*)-stereochemistry has a potency of 350, and (−)-curarine (25) with the doubly unfavoured (*R,R*)-configuration has a potency of only 130 relative to tubocurarine (21) [89].



23



24



25

A TENTATIVE MODEL FOR RECEPTOR INTERACTIONS

Any model proposed to account for receptor response should ideally be capable of providing a rational explanation of both the agonistic effects of acetylcholine and the antagonist effects of neuromuscular blocking agents and neurotoxic venoms. In particular, it must endeavour to account for the following phenomena:

(a) agonist-receptor and antagonist-receptor affinity;
(b) triggering of the consequential changes in receptor conformation;
(c) the opening of ion channels with the observed differential rates of ion flow across the post-synaptic membrane in response to acetylcholine;
(d) observed differences in response to blockade by depolarising and non-depolarising neuromuscular blocking agents and neurotoxic venoms.

The present state of knowledge is such that it is still only possible to speculate and suggest tentative mechanisms which provide an acceptable explanation of some of these phenomena. The proposals outlined in the sequel are put forward in an endeavour to create a rational synthesis of mechanistic events based on a wide spectrum of observations. They are, admittedly, speculative and only time and further experiment will either confirm or displace them.

There can be little doubt that the evidence available strongly implicates ionic bonding of the quaternary ammonium cation of acetylcholine and neuromuscular blocking agents with the free carboxylate anion of an aspartate or glutamate residue as the primary binding mechanism. The agonist response consisting of a change in receptor conformation and ion flow is observed only in response to acetylcholine and the depolarising antagonists, decamethonium ($Me_3N^+(CH_2)_{10}\cdot N^+Me_3\ 2I^-$) and suxamethonium (12, $R^1 = R^2 = R^3 = H$), all of which have trimethylammonium ($Me_3N^+ -$) groups in common. Increase in overall quaternary ammonium head group size as in the series of alkyltrimethylammonium compounds ($R\cdot N^+Me_3X^-$), in decaethonium ($Et_3N^+(CH_2)_{10}\cdot N^+\cdot Et_3\ 2I^-$) and gallamine triethiodide [90,91], which, incidentally, also increases affinity by hydrophobic interaction at an adjacent subsite [42—45], leads to compounds which are non-depolarising. It appears, therefore, that generation of the agonist response requires not only close affinity, but close range ionic bonding, and because of steric considerations, this is only feasible in trimethylammonium compounds. Steric restriction to access at the receptor cation-binding anionic subsite also provides a logical explanation of the importance of lysine residues

[$H_3N^+(CH_2)_4 \cdot CH(NH—) \cdot CO—$] in the snake neurotoxins, and, likewise, both the complete lack of neurotoxicity when they are blocked by carbamoylation [$HCO \cdot NH(CH_2)_4 \cdot CH(NH—) \cdot CO—$] and the retention of toxicity at a lower level when they are converted to larger homoarginine residues [$HN = C(N^+H_3) \cdot NH(CH_2)_4 \cdot CH(NH—) \cdot CO—$].

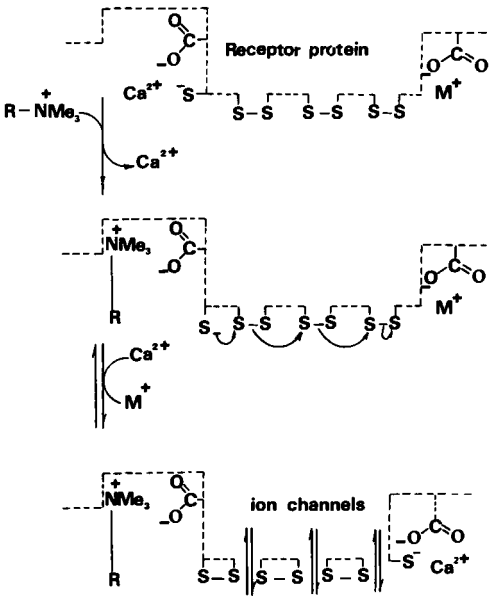
Evidence based on autoradiographic studies with radiolabelled *α*-bungarotoxin puts average receptor density at approximately one per 5000 $Å^2$ of the post-synaptic membrane in mouse, rat, chick and rhesus monkey [92], giving an average distance between receptors of ca. 70 Å. Even allowing for uneven distribution and concentration of receptors in particular areas, it is unlikely that the much greater potency of bisquaternary neuromuscular blocking agents compared with monoquaternary compounds is due to their ability to span receptors. Likewise, although it is established that decamethonium and snake neurotoxins are capable of cross-linking receptor sub-units and partially inhibiting dissociation of the receptor protein [21,25,26], it is also well-known that the action of depolarising neuromuscular blocking agents such as decamethonium and suxamethonium is characterised by a typical initial agonist response involving muscular twitching and depolarisation. It seems evident, therefore, that the agonist response is not initiated by any dissociation of the receptor protein into sub-units, but rather reaction to the close antagonist association at the receptor anionic subsite which is characteristic of these compounds.

A possible clue to the mechanism of this reaction may be in the similarity between structural components of the snake neurotoxins and the receptor subsite. Thus, all the snake toxins have their strongly basic lysine and/or arginine residues in close juxtaposition to a Cys-Cys disulphide bridge. Likewise, the receptor protein has either or both a free sulphydryl and one or more disulphide bonds within 9 to 12 Å of the receptor cation-binding subsite. Is it possible that agonist-receptor binding involves release of a masked receptor sulphydryl group, which in agonist activity opens ion channels by triggering a cascade of sulphydryl-disulphide displacements with concomitant changes in receptor conformation? If so, such reactions would also be blocked by appropriately-sited disulphide groups in the snake neurotoxins. Moreover, it would not be out of keeping with earlier speculations of Chang, Lee, Wang and Chuang [93] who, having observed that the thiol reagent phenylmercuric acetate caused a fall in membrane potential and development of contracture in rat diaphragm, were led to suggest that 'sulphydryl groups determine the necessary conformational structure of macromolecules which participate in impulse conduction in axonal membranes'.

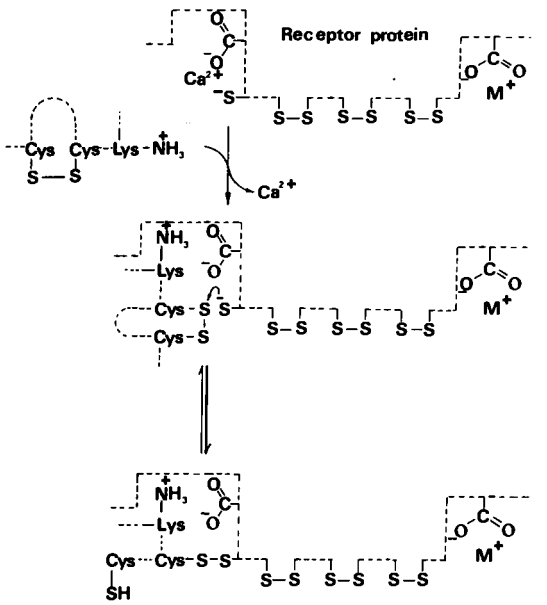
Acetylcholine action causes calcium release from the subsynaptic mem-

brane [67]. This has been demonstrated by formation of a 'lake' with the dyestuff alizarin red S, characteristic of divalent cations, which is formed in the subsynaptic area in response to nerve stimulation and by the action of depolarising neuromuscular blocking agents, but not by non-depolarising compounds [94,95]. The displacement of Ca^{2+} from, say, a hard acid-hard base-soft acid [98—100] involving both carboxylate and sulphydryl bonds would readily explain why only the most strongly basic cations (quaternary ammonium and lysyl) are capable of functioning in neuromuscular blockade. Such a displacement would also lead to the formation of a much less stable hard acid-soft base complex from which both Ca^{2+} and the sulphydryl ion would then be free to dissociate, the former to enhance ionic strength and assist moderation of receptor conformation, and the latter to interact with disulphide bonds and stabilise a new conformation permitting ion flow, as tentatively represented in *Scheme 5.3.*

The ionic imbalance of around 3000 univalent cations per acetylcholine molecule released [7] represents a massive change in ionic strength on both sides of the membrane, which on falling to a critical level, would facilitate



Scheme 5.3. A tentative mechanism for agonist/depolarising antagonist-receptor interaction leading to opening of ion channels.



Scheme 5.4. A tentative mechanism for snake neurotoxin-receptor interaction.

dissociation of the antagonist from the receptor and trigger a further sequence of events which would restore the initial protein conformation. Interruption of the depolarisation process by snake neurotoxins would involve formation of a disulphide bond between toxin and receptor, as represented in *Scheme 5.4.*

Such a mechanism would explain blockage of the neuromuscular junction by sulphydryl reagents [32,33] and, specifically, their inhibitory effect on ion flow [99,100]. Several aspects of recent work are, likewise, consistent with the concept of what has been described as a Ca^{2+}-binding subsite at the acetylcholine receptor [101]. Thus, whilst it has been reported that acetylcholine receptor-rich fragments of *T. marmorata* showed increased affinity for cholinergic ligands in the presence of Ca^{2+} [102], Eldefrawi, Eldefrawi, Penfold, O'Brien and Van Campen [103] have shown that Ca^{2+} actually competes with ACh at purified receptor proteins from *T. Californica.* Likewise, calcium ions compete with the fluorescent cholinergic ligand, bis(3-aminopyridinium)-1,10-decane at *T. Californica* receptors [104]. Further work has also shown both that the fluorescent lanthanide, Tb^{3+}, competes with Ca^{2+} at receptor-binding sites [103] and that it can be used to

characterise the properties of the Ca^{2+}-binding subsite. Studies using both the lanthanide probe and ^3H-labelled MBTA as affinity reagent show that ACh receptors for *T. ocellata* have four sub-units [101]. Both the affinity label binding site and the Tb^{3+}-binding site are associated with the sub-unit of mol. wt. 40 000, but, whereas affinity for Tb^{3+} is the same in both the intact tetramer and the sub-unit, affinity for Ca^{2+} decreases 4-fold in the sub-unit. Hydrolysis of receptor protein with trypsin and chymotrypsin to peptides of mol. wt. 8000 or less does not affect the Tb^{3+}-binding site. The authors, therefore, conclude that the behaviour of the Tb^{3+} and Ca^{2+}-binding sites reflect structural properties rather than chain folding of the receptor protein.

Lastly, we must consider whether the hypothesis can accommodate non-depolarising as well as depolarising neuromuscular blocking agents. Is the difference merely one of molecular fit, primarily at the cation-binding anionic subsite? Is the ionic bonding of depolarising agents, all of which have at least one N^+-methyl substituent, able to permit sufficiently close approach to displace Ca^{2+} and form a new ionic bond, stabilised by secondary (hydrophobic) bonding forces? And, does depolarisation fail to materialise because the much larger and more complex cations of the non-depolarisers merely associate electrostatically with the anionic subsite in competition with Ca^{2+}, but as observed fail to displace it because of steric considerations [105]. If so, why is the second (and third) cationic component so important for the potency of non-depolarising neuroblockers as evidenced by its potency-enhancing effects in tubocurarine, pancuronium, gallamine and other clinically-useful agents compared with their related monoquaternary derivatives? Does the second cationic centre merely stabilise antagonist-receptor association by sub-unit cross-linking as observed with the bisquaternary compound, bis(3-aminopyridinium)-1,10-decane [104], decamethonium and *N. nigricollis* α-toxin [21,24], and in so doing, simply deny access of acetylcholine to the cation-binding subsite? The answer to these questions must surely lie in further experiment.

REFERENCES

1. M. Martin-Smith in: Rational Elements in the Development of Superior Neuromuscular Blocking Agents in Drug Design, ed. E.J. Ariëns (Academic Press, London, 1971) Vol. II, pp. 454-530.
2. J.I. Hubbard, Physiol. Rev., 53 (1973) 674.
3. J.I. Hubbard and D.M.J. Quastel, Annu. Rev. Pharmacol., 13 (1973) 199.
4. J.B. Stenlake, Neuromuscular Blocking Agents, in: Alfred Burger's Medicinal Chemistry, ed. M.E. Wolff (Wiley-Interscience, New York) 4th Edn., in press.

5. E.M. Kosower and R. Werman, Nature (New Biol.) (London), 233 (1971) 121.
6. R.W. Abers and G.J. Koval, Biochim. Biophys. Acta, 60 (1962) 359.
7. S.W. Kuffler and D. Yoshikami, J. Physiol. (London), 251 (1975) 465.
8. J.C. Eccles, B. Katz and S.W. Kuffler, J. Neurophysiol., 4 (1941) 363.
9. P. Fatt and B. Katz, J. Physiol. (London), 115 (1951) 320.
10. H.A. Lester, Sci. Am., 236 (1977) 106.
11. C. Chargas, E.P. Franca, K. Nishie and E.J. Garcia, Arch. Biochem. Biophys., 76 (1958) 251.
12. S. Ehrenpreis, Biochim. Biophys. Acta, 44 (1960) 561.
13. J.L. La Torre, G.S. Lunt and E. DeRobertis, Proc. Nat. Acad. Sci. U.S.A., 65 (1970) 716.
14. R. Miledi, P. Molinoff and L.T. Potter, Nature (London), 229 (1971) 554.
15. R. Miledi and L.T. Potter, Nature (London), 233 (1971) 599.
16. D.K. Berg, R.B. Kelly, P.B. Sargent, P. Williamson and Z.W. Hall, Proc. Nat. Acad. Sci. U.S.A., 69 (1972) 147.
17. K. Narita, D. Mebs, S. Iwanaga, Y. Samejima and C.Y. Lee, Biochem. Biophys. Res. Commun., 44 (1971) 711.
18. D. Mebs, K. Narita, S. Iwanaga, Y. Samejima and C.Y. Lee, Hoppe-Seyler's Z. Physiol. Chem., 353 (1972) 243 (from Chem. Abstr., 76 (1972) 109380).
19. C.Y. Lee, S.L. Chang, S.T. Kau and S.-H. Luck, J. Chromatogr., 72 (1972) 71.
20. R.W. Olsen, J.C. Meunier and J.P. Changeux, FEBS Lett., 28 (1972) 96.
21. F. Hucko and J.P. Changeux, FEBS Lett., 38 (1973) 11.
22. J.C. Meunier, R.W. Olsen, A. Menez, P. Fromageot, P. Boquet and J.P. Changeux, Biochemistry, 11 (1972) 1200.
23. M.J. Reiter, D.A. Cowburn, J.M. Prives and A. Karlin, Proc. Nat. Acad. Sci. U.S.A., 69 (1972) 1168.
24. G.E. Davies and G.R. Stark, Proc. Nat. Acad. Sci. U.S.A., 66 (1970) 651.
25. J.C. Meunier, R. Sealock, R. Olsen and J.P. Changeux, Eur. J. Biochem., 45 (1974) 371.
26. J. Schmidt and M.A. Raftery, Biochemistry, 12 (1973) 852.
27. C.L. Weill, M.G. McNamee and A. Karlin, Biochem. Biophys. Res. Commun., 61 (1974) 997.
28. M.E. Eldefrawi, A.T. Eldefrawi and D.B. Wilson, Biochemistry, 14 (1975) 4304.
29. J.P. Changeux, T.R. Podlexki and J.C. Meunier, J. Gen. Physiol., 54 (1969) 225.
30. R.M. Krupka, Biochemistry, 5 (1966) 1988.
31. R.M. Krupka, Biochemistry, 6 (1967) 1183.
32. J. Del Castillo and B. Katz, J. Physiol. (London), 128 (1955) 157.
33. A. Karlin and E. Bartels, Biochem. Biophys. Acta, 126 (1966) 525.
34. W.W. Cleland, Biochemistry, 3 (1964) 480.
35. G.L. Ellman, Arch. Biochim. Biophys., 82 (1959) 70.
36. A. Karlin and M. Winnik, Proc. Nat. Acad. Sci. U.S.A., 60 (1968) 668.
37. A. Karlin, J. Gen. Physiol., 54 (1969) 245S.
38. T. Podleski and D. Nachmansohn, Proc. Nat. Acad. Sci. U.S.A., 56 (1966) 1034.
39. P.H. Elworthy, J. Pharm. Pharmacol., 15 (1963) 137T.
40. J. Patrick, B. Valeur, L. Monneric and J.B. Changeux, J. Membrane Biol., 5 (1971) 102.
41. H.G. Mautner, E. Bartels and G.D. Webb, Biochem. Pharmacol., 15 (1966) 187.
42. H.R. Ing and W.M. Wright, Proc. R. Soc. Ser. B. 109 (1931) 337.
43. M.J. Dallemagne and E. Phillipott, Arch. Int. Pharmacodyn., 87 (1951) 127.
44. J. Thomas and G.A. Starmer, J. Pharm. Pharmacol., 13 (1961) 752.
45. T.R. Podleski, Biochem. Pharmacol., 18 (1969) 211.

46. K. Nakai, C. Nakai, T. Sasaki, K. Kakiuchi and R. Hayashi, Naturwissenschaften, 57 (1970) 387.
47. E. Karlson, D. Eaker and G. Ponterius, Biochem. Biophys. Acta, 257 (1972) 235.
48. H. Kyozo, N. Kenji, T. Sasaki and T. Suzuki, Biochem. Biophys. Res. Commun., 36 (1969) 482.
49. D.P. Botes and D.J. Strydom, J. Biol. Chem., 244 (1969) 4147.
50. D.P. Botes, D.J. Strydom, C.G. Anderson and P.A. Christensen, J. Biol. Chem., 246 (1971) 3132.
51. D.P. Botes, J. Biol. Chem., 246 (1971) 7383.
52. B.V. Hofstén, H. van Klev and D. Eaker, Biochem. Biophys. Acta, 110 (1965) 585.
53. C.C. Yang, Biochim. Biophys. Acta, 133 (1967) 346.
54. C.C. Yang, J. Biochem. (Tokyo), 61 (1967) 272. (from Chem. Abstr., 66 (1967) 101872).
55. L.T. Potter, J. Physiol. (London), 206 (1970) 145.
56. J.I. Hubbard and D.F. Wilson, J. Physiol (London), 228 (1973) 307.
57. D.M. Farnbrough and H.C. Hartzell, Science, 176 (1972) 189.
58. P.G. Waser, Ann. N.Y. Acad. Sci., 144 (1967) 737.
59. A.G. Ogston, J. Physiol. (London), 128 (1955) 222.
60. J.C. Eccles and M.V. Macfarlane, J. Neurophysiol., 12 (1949) 59.
61. M. Kordas, J. Physiol. (London), 224 (1972) 333.
62. D. Nachmansohn, Chemical and Molecular Basis of Nerve Activity (Academic Press, New York, 1959).
63. A.O. Župančič, Ann. N.Y. Acad. Sci., 144 (1967) 689.
64. C.C. Chang, J. Formosan Med. Assoc., 59 (1960) 416.
65. C.C. Chang, S.E. Lu, P.N. Wang and S.T. Chuang, Eur. J. Pharmacol., 11 (1970) 195.
66. Z.W. Hall and R.B. Kelly, Nature (New Biol.) (London), 237 (1971) 62.
67. B. Csillick, Acta Physiol. Acad. Sci. Hung., 26 (1964) 327.
68. C. Van der Meer, Nature (London), 171 (1953) 78.
69. R.J. Kitz and L.T. Kremzner, Molec. Pharmacol., 4 (1968) 104.
70. J.B. Stenlake, W.D. Williams, N.C. Dhar and I.G. Marshall, Eur. J. Med. Chem., 9 (1974) 233.
71. J.B. Stenlake, W.D. Williams, N.C. Dhar, R.D. Waigh and I.G. Marshall, Eur. J. Med. Chem., 9 (1974) 243.
72. A. Simonart, J. Pharmacol. Exp. Ther., 46 (1932) 157.
73. C.C.J. Culvenor and N.S. Ham, Chem. Commun., 15 (1966) 537.
74. A.H. Beckett, Ann. N.Y. Acad. Sci., 144 (1967) 677.
75. V. Rosnati, Gazz. Chim. Ital., 80 (1950) 663.
76. E. Lesser. J. Pharm. Pharmacol., 13 (1961) 703.
77. E. Lesser, Ph.D. Thesis, London University (1967).
78. A.H. Beckett, N.J. Harper and J.W. Clitherow, J. Pharm. Pharmacol., 15 (1963) 349.
79. A.H. Beckett, C.L. Vaughan and M. Mitchard, J. Med. Chem., 11 (1968) 1126.
80. P.D. Armstrong, J.G. Cannon and J.P. Long, Nature (London), 220 (1968) 65.
81. C.Y. Chio, J.P. Long, J.G. Cannon and P.D. Armstrong, J. Pharm. Exp. Ther., 166 (1969) 243.
82. B.A. Hemsworth and J.B. Robinson, British Pharmaceutical Conference Communication 1977, unpublished.
83. J.B. Stenlake and N.C. Dhar, Eur. J. Med. Chem., 13 (1978) 343.
84. A.R. Ubbelohde, Trans. Farad. Soc., 32 (1936) 525.
85. K. Mislow, R. Grieve, H.J. Gordon and G.H. Wahl, J. Am. Chem. Soc., 86 (1964) 1733.

86. A. Horeau, A. Nouaille and K. Mislow, J. Am. Chem. Soc., 87 (1965) 4957.
87. T.O. Soine and J. Naghaway, J. Pharm. Sci., 63 (1975) 1643.
88. O. Wintersteiner, in: Curare and Curare-like Agents, eds. D. Bovet, F. Bovet-Nitti and G.B. Marini-Bettôlo (Elsevier, London, 1959), 160.
89. H.A. Holiday and R.F. Varney, quoted by O. Wintersteiner, Ref. 88.
90. D. Bovet, F. Depierre and Y. De Lestrange, C.R. Acad. Sci., Paris, 225 (1947) 74.
91. D. Bovet, F. Depierre, S. Courvoisier and Y. De Lestrange, Arch. Int. Pharmacodyn., 80 (1949) 172.
92. E.A. Barnard, J. Wieckowski and T.H. Chiu, Nature (London), 234 (1971) 207.
93. C.C. Chang, S.E. Lu, P.N. Wang and S.T. Chuang, Eur. J. Pharmacol., 11 (1970) 195.
94. M. Lievramont, M. Czajka and F. Tazieff-Depierre, C.R. Acad. Sci., Paris, 267D (1968) 1988.
95. M.Lievramont, M. Czajka and F. Tazieff-Depierre, C.R. Acad. Sci., Pairs, 268D (1969) 379.
96. R.G. Pearson, J. Am. Chem. Soc., 85 (1963) 3533.
97. R.G. Pearson, Science, 151 (1966) 172.
98. R.G. Pearson, J. Chem. Ed., 45 (1968) 581, 643.
99. W.G. Van der Kloot, J. Gen. Physiol., 41 (1958) 879.
100. H.S. Frazier and R.D. Keynes, J. Physiol. (London), 148 (1959) 362.
101. H. Rübsamen, M. Montgomery, G.P. Hess, A.T. Eldefrawi and M.E. Eldefrawi, Biochem. Biophys. Res. Commun., 70 (1976) 1020.
102. J.B. Cohen, M. Weber and J.-P. Changeux, Mol. Pharmacol., 10 (1974) 904.
103. M.E. Eldefrawi, A.T. Eldefrawi, L.A. Penfold, R.D. O'Brien and D. Van Campen, Life Sciences, 16 (1965) 925.
104. M. Martinez-Carrion and M.A. Raftery, Biochem. Biophys. Res. Commun., 55 (1973) 1156.
105. H. Rübsamen, G.P. Hess, A.T. Eldfrawi and M.E. Eldfrawi, Biochem. Biophys. Res. Commun., 68 (1976) 56.
106. B.W. Low, H.S. Preston, A. Sata, L.S. Rosen, J. E. Searl, A.D. Rudko and J.S. Richardson, Proc. Natl. Acad. Sci., U.S.A., 73 (1976) 2991.
107. D. Tsernoglou and G. Petsko, FEBS Lett., 68 (1976) 1.
108. M.J. Dufton and R.C. Hider, J. Mol. Biol., 115 (1977) 177.
109. A.F. Drake, M.J. Dufton and R.C. Hider, FEBS Lett., 83 (1977) 202.
110. T.-H. Hseu, Y.-C. Liu, C. Wang, H. Chang, D.-M. Hwang and C.-C. Yang, Biochemistry, 16 (1977) 2999.
111. A.S. Arseniev, T.A. Balashova, Y.N. Utkins, V.I. Tsetlin, V.F. Bystrov, V.T. Ivanov and Y.A. Ovchinnikov, Eur. J. Biochem., 71 (1976) 595.

NOTE ADDED IN PROOF

More recent evidence of the tertiary structure of snake neurotoxins comes from the X-ray studies of erabutoxin b [106] and a neurotoxin from the sea snake *Laticauda semifasciata* [107], from conformational prediction studies [108–110], and from NMR data [111]. These show that both the short and long neurotoxins share common tertiary structures, with a common shape consisting of extensive β-sheet elements with a β-turn based on either histidine or phenylalanine at 32, giving a common mode of action. It is concluded [109] that the residues Lys^{47}, Arg^{33}, Trp^{29} and Phe^{32}/His^{32} common to all short neurotoxins (ca 60 amino acid residues) form a close stereochemical mimic of tubocurarine.

Index